A STUDY OF THE ROLE OF SPLENIC MESENCHYME-TO-EPITHELIAL TRANSITION IN ISLET NEOGENESIS

Stuart Robertson

Paediatric Surgical Research Laboratory
Nuffield Department of Surgery
University of Oxford

MD student
College of Medicine and Veterinary Medicine
University of Edinburgh

MD supervisors:

Professor Paul Johnson
Professor of Paediatric Surgery
University of Oxford

Professor Robert Minns
University of Edinburgh

Thesis presented to the University of Edinburgh in November 2008
for the degree of Doctor of Medicine (MD)

"I am the vine, ye are the branches:
He that abideth in me, and I in him,
the same bringeth forth much fruit"

John 15 : 5 KJV

Thesis declaration

I declare that this thesis has been composed by myself and that the work contained is my own. This work has not been submitted for any other degree or professional qualification.

Signed:

Stuart Robertson

Abstract

Type 1 Diabetes Mellitus (T1DM) affects millions of children worldwide and is increasing in prevalence. Exogenous insulin therapy is currently the mainstay of treatment but is unable to prevent the chronic complications of this disease. Islet transplantation is a successful, minimally-invasive, potentially curable alternative treatment, which has restored physiological euglycaemia in up to 85% of recipients in recent clinical trials. However, worldwide human donor islet shortages limit the wider application of this treatment. Pluripotent cells may provide alternative islet sources to overcome this shortage. The human spleen may be one such source and is an excellent candidate tissue for further investigation.

The main aims of this thesis were to investigate whether the developing spleen could differentiate into insulin-producing cells and to investigate the molecular mechanisms behind this. Using an avian model of pancreatic development, I characterise normal avian foregut expression of the splenic mesenchymal transcription factor *Tlx-1* between E4-E11 of development and report an optimised *in situ* hybridisation protocol for this. I use a chick-quail chimaera model of pancreatic organogenesis to show that the developing avian spleen is able to differentiate into insulin-producing cells *in vitro* through islet Mesenchyme-to-Epithelial Transition (iMET). I show evidence that, when recombined with differentiating pancreatic epithelium, splenic mesenchyme is reprogrammed to express the pancreatic islet genes *Pdx-1* and *Isl-1*. *Tlx-1* is dramatically down-regulated during this process, indicating that this tissue is reprogrammed from a splenic to pancreatic endocrine fate. Finally, an attempt to augment splenic iMET is made through the addition of a *Wnt* agonist.

These findings, together with the recent discovery that the mature human spleen contains *Tlx-1* positive cells, may be a useful target for future bench-to-bedside translation strategies for this work. Therefore, the spleen may be an ideal future tissue source for islet transplantation to treat patients with T1DM.

Abbreviations

ALL	acute lymphoblastic leukaemia
ARX	aristaless related homeobox
Bapx	bagpipe homeobox factor
Barx	BarH-like homeobox
BCIP	5-bromo-4-chloro-3-indolyl phosphate, toluidine salt
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
Brn	brain-specific homeobox
CD	cluster of differentiation
Cdx	caudal type homeobox
CGMS	continuous glucose monitoring system
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CITR	Collaborative Islet Transplant Registry
CMF	calcium magnesium free
Cmyc	cellular myelocytomatosis oncogene
CO_2	carbon dioxide
DAB	3,3-diaminobenzidine
DAPI	4,6-diaminobenzidine tetrahydrochloride
DCCT	Diabetes Control and Complications Trial
DCCTRG	Diabetes Control and Complications Trial Research Group
dH_20	distilled water
ddH_20	double-distilled water
DIG	digoxigenen
DNA	deoxyribonucleic acid
DPE	dorsal pancreatic epithelium
DPM	dorsal pancreatic mesenchyme
DPX	distyrene plasticizer xylene mixture
DSHB	developmental studies hybridoma bank
E	embryonic day
ECM	extracellular matrix
EDICSG	Epidemiology of Diabetes Interventions and Complications Study Group
EGTA	ethylene glycol tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay

EMT	Epithelial-to-Mesenchyme Transition
ES	embryonic stem
FCA	Freund's complete adjuvant
FGF	fibroblast growth factor
FISH	fluorescent *in situ* hybridisation
Fox	forkhead box
Fz	Frizzled
GFP	green fluorescent protein
Glut2	glucose transporter type 2
GSK	glycogen synthase kinase
GVHD	graft-versus-host disease
HbA1C	glycosylated haemoglobin
Hes	hairy and enhancer of split
HH	Hamburger-Hamilton stage
Hlx	H2.0-like homeobox
Hnf	hepatocyte nuclear factor
Hox	homeo box
HVGD	host-versus-graft disease
iMET	islet Mesenchyme-to-Epithelial Transition
IPC	insulin-producing cell
Isl	islet
IA	insulinoma-associated
Klf	Kruppel-like factor
LB	Luria-Bertani
Lef	lymphoid-enhancer binding factor
LPM	lateral plate mesoderm
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene
MET	Mesenchyme-to-Epithelial Transition
MHC	major histocompatibility complex
MIP	mouse insulin promoter
mPygo	pygopus
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
NBT	nitro blue tetrazolium chloride
Ngn	neurogenin
NICHD	National Institute of Child Health and Human Development
Nkx	NK homeobox

NOD	non-obese diabetic
Onecut	one cut domain
Oct	octamer
OCT	optimum cutting temperature
OPSI	overwhelming post-splenectomy infection
Pax	paired homeobox
PERV	porcine endogenous retrovirus
PBS	phosphate-buffered saline
PBT	phosphate-buffered saline with Triton-X
Pbx	pre-B-cell leukaemia homeobox
Pdx	pancreas duodenum homeobox
PFA	paraformaldehyde
Pod	podocyte-expressed
PP	pancreatic polypeptide
Ptf	pancreas transcription factor
PVA	poly vinyl alcohol
QCPN	quail not chick perinuclear
RA	retinoic acid
Rpm	revolutions per minute
RT	room temperature
Shh	sonic hedgehog
SNP	single nucleotide polymorphism
Sox	(sex determining region Y)-box
SRTR	Scientific Registry of Transplant Recipients
SSC	3M sodium chloride, 300mM tri-sodium citrate dihydrate
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
Tcf	T-cell specific transcription factor
TGF	transforming growth factor
Tlx	T-cell leukaemia homeobox
TUNEL	terminal transferase uridyl nick end labelling
VPE	ventral pancreatic epithelium
VPM	ventral pancreatic mesenchyme
WHO	World Health Organisation
Wnt	wingless-type
Wt	Wilms tumour suppressor gene

Contents

Page

List of figures

List of tables

SECTION ONE

Introductory chapters

Chapter One

Pancreatic islet transplantation – rationale, results and current challenges

1.1 The rationale for pancreatic islet transplantation

Diabetes Mellitus is the commonest human metabolic disorder, affecting over 200 million people worldwide and increasing in prevalence (King, Aubert et al. 1998; Wild, Roglic et al. 2004). This condition is characterised by a critical reduction in the functional beta-cell mass of the pancreas (Donath and Halban 2004; Maedler 2008), resulting in hyperglycaemia and abnormal glucose homeostasis through reduced insulin secretion. Around 10% of cases are due to *Type 1 Diabetes Mellitus* (T1DM), also known as "Insulin-Dependent Diabetes Mellitus" or "Juvenile-Onset Diabetes Mellitus". Incredibly, the incidence of childhood onset T1DM has doubled over the past 10 years (Soltesz, Patterson et al. 2007). In T1DM, targeted autoimmune destruction of beta-cells (Soeldner, Tuttleman et al. 1985; Atkinson and Maclaren 1994; Atkinson and Eisenbarth 2001) results in a 90% loss of functional beta-cell mass (Kloppel, Drenck et al. 1984; Lohr and Kloppel 1987; Hanafusa, Miyazaki et al. 1990; Pipeleers and Ling 1992). The pathophysiology of *Type 2 Diabetes Mellitus* (T2DM) is complex and multifactorial, involving a genetic predisposition, a reduced functional beta-cell mass and an increase in insulin resistance (Gerich 1999; Bouwens and Rooman 2005; Leahy 2005; Freeman and Cox 2006). However, in the later stages of T2DM, beta-cell mass is reduced by around 50-65%. This lesser reduction is via a ten-fold increase in beta-cell apoptosis instead of autoimmune destruction (Clark, Wells et al. 1988; Butler, Janson et al. 2003; Kharroubi, Ladriere et al. 2004; Marchetti, Del Guerra et al. 2004). Approximately 20-30% of patients with T2DM require exogenous insulin therapy, when they no longer respond to oral hypoglycaemic agents.

The Nobel prize-winning discovery of insulin by Banting and Best in 1922 heralded a new era in diabetes treatment (Banting and Best 1922; Banting, Best et al. 1922). Prior to this, patients with T1DM would die from diabetic ketoacidosis and typically at an early age. Patients could now survive by using exogenous insulin therapy. However, it later became apparent that despite regular insulin therapy, an increasing number of patients developed a range of long-term complications of the disease (Kimmelstiel and Wilson 1936; Bell 1942; White and Waskow 1948). Diabetic complications are now a leading cause of early mortality and severe morbidity (Manuel and Schultz 2004). Hyperglycaemic diabetic complications (see Figure 1) include *macrovascular* complications, such as coronary artery disease (with subsequent ischaemic heart disease), cerebral vascular accidents and peripheral vascular disease (with amputations), and *microvascular* complications, such as retinopathy (and subsequent blindness), nephropathy (with end-stage renal failure) and neuropathy. Hypoglycaemic complications, from insulin therapy and some oral hypoglycaemic agents, include hypoglycaemic unawareness and subsequent loss of consciousness, or even death. The complications of diabetes, combined with the increase in incidence of types 1 and 2 (Passa 2002), pose an enormous health and financial burden on society, prompting research into strategies to reduce this morbidity and mortality. In particular, the relationship between glycaemic control and the onset of diabetic complications has been extensively investigated.

Figure 1 Examples of diabetic retinopathy and diabetic nephropathy

Fig 1(a)

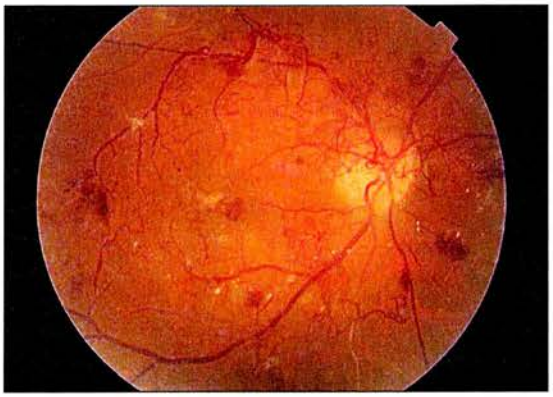


Fig 1(a) Retinal photograph showing diabetic proliferative retinopathy (Yorston 2003)

Fig 1(b)

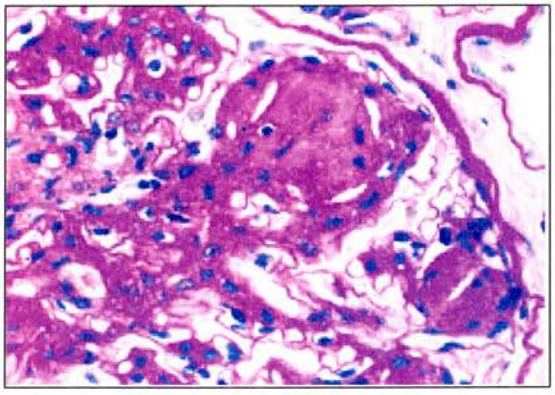


Fig 1(b) Example of glomerulosclerosis in diabetic nephropathy; original image x400 (Paueksakon, Revelo et al. 2002)

The *Diabetes Control and Complications Trial* (DCCT) was a multicentre randomised controlled trial designed to compare intensive with conventional diabetes therapy, with regard to their effects on the development and progression of diabetic complications in patients with T1DM (DCCTRG 1993). A total of 1441 patients were recruited: 726 patients had no retinopathy at baseline (the primary-prevention cohort) and 715 patients had mild retinopathy at baseline (secondary-intervention cohort). Patients were randomly assigned to receive either intensive insulin therapy (administered by three or more daily insulin injections, or by an insulin pump, and guided by frequent blood glucose monitoring) or to conventional therapy (with one or two daily insulin injections). The DCCT confirmed a relationship between T1DM complications and both glycaemic control and duration of disease. Furthermore, it showed that tight glycaemic control achieved by intensive insulin therapy can reduce the incidence of chronic complications (see Figures 2 and 3). In patients with no retinopathy at enrolment into the DCCT, intensive therapy reduced the adjusted mean risk for the development of retinopathy by 76% (95 percent confidence interval, 62 to 85%), as compared with conventional therapy. In patients with mild retinopathy at enrolment, intensive therapy slowed the progression of retinopathy by 54% (95 percent confidence interval, 39 to 66%) and reduced the development of proliferative or severe nonproliferative retinopathy by 47% (95 percent confidence interval, 14 to 67%). In the two cohorts combined, intensive therapy reduced the occurrence of microalbuminuria (urinary albumin excretion of ≥40 mg per 24 hours) by 39% (95 percent confidence interval, 21 to 52%), that of albuminuria (urinary albumin excretion of ≥300 mg per 24 hours) by 54% (95 percent confidence interval, 19 to 74%), and that of clinical neuropathy by 60% (95 percent confidence interval, 38 to 74%). The DCCT was stopped prematurely after a mean follow-up time of 6.5 years,

when the benefits of intensive treatment were deemed incontrovertible (EDICSG 2002). However, intensive insulin therapy did not restore true physiological euglycaemia and the benefits of tight control came with the associated two-to-threefold increased risk of potentially life-threatening severe hypoglycaemic episodes (DCCTRG 1997; EDICSG 2002; EDICSG 2003; Nathan, Cleary et al. 2005).

Figure 2 Retinopathy in patients with T1DM receiving intensive or conventional therapy

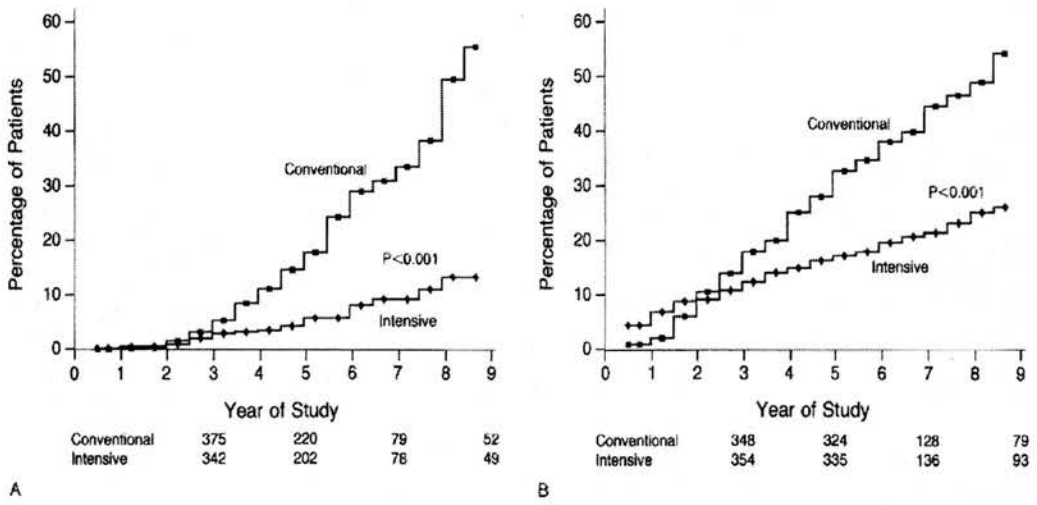


Fig 2 Cummulative incidence of a sustained change in retinopathy in patients with T1DM receiving intensive or conventional therapy; Panel A = primary–prevention cohort, Panel B = secondary-intervention cohort (DCCTRG 1993)

Figure 3 Urinary Albumin Excretion in patients with T1DM receiving intensive or conventional therapy

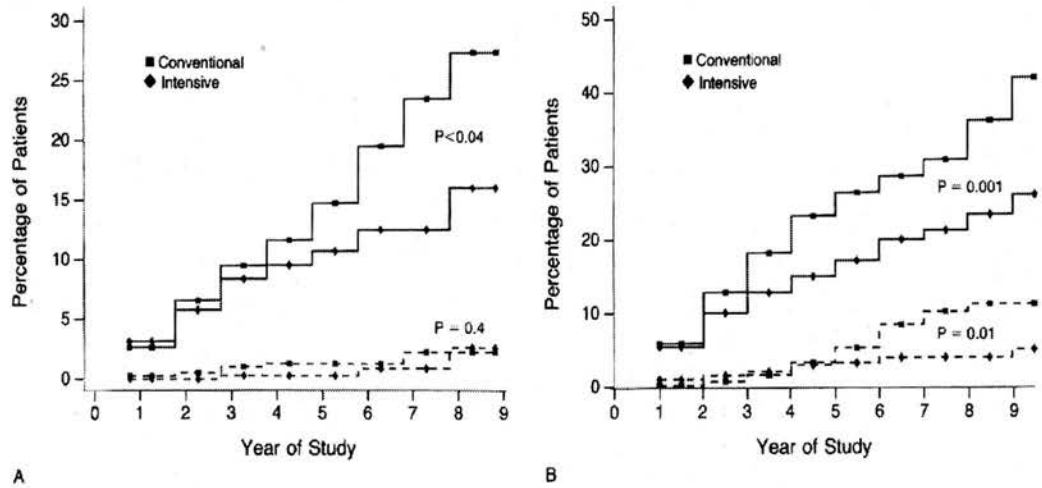


Fig 3 Cumulative Incidence of Urinary Albumin Excretion ≥300 mg per 24 Hours (Dashed Line) and ≥40 mg per 24 Hours (Solid Line) in Patients with IDDM Receiving Intensive or Conventional Therapy; Panel A = primary–prevention cohort, Panel B = secondary-intervention cohort (DCCTRG 1993)

The DCCT provided clear evidence of the benefits of maintaining blood glucose concentrations as close to the normal range as possible. Therefore, therapeutic strategies have subsequently focussed on ways to improve glycaemic control, such as the more widespread use of the mechanical insulin delivery pump. This device uses a programmable pump and insulin storage reservoir to provide a continuous supply of subcutaneous insulin, and the patient can trigger infusion pulses to cover meals (Tamborlane 2006). Improvements in technology and the desire to achieve optimal blood glucose control have resulted in a sharp increase in their use. However, such mechanical devices require patient competency and motivation to use the device, and carry the risk of pump malfunction (mechanical failure), prosthesis-related sepsis, and concerns over non-physiological insulin pump delivery. The recent introduction of continuous glucose monitoring systems (CGMSs) may offer the prospect of improving insulin pump therapy to normalize HbA1C levels with minimal risk of hypoglycaemia (Ellis, Naik et al. 2008). However, at present patients need to regularly calibrate the subcutaneous CGMS devices with their blood glucose meter measurements. Currently, they are therefore a useful adjunct for detecting blood glucose fluctuations, but not yet a replacement for blood glucose meter measurements. Biomedical engineering research continues into ways of safely and effectively integrating the CGMS and insulin pump together to create an electronic "artificial pancreas" (Gomez, Hernando Perez et al. 2008). Some of the concerns over insulin pump therapy have been addressed through recent innovations, including a reduced incidence of catheter obstructions through improved catheter design (Renard and Schaepelynck-Belicar 2007). However, these pumps cannot yet achieve true euglycaemia, and therefore an ideal treatment for T1DM is still sought. The ideal treatment therapy for T1DM would restore true physiological euglycaemia shortly

after diagnosis (and so would need to be applicable for use in children) and would be widely available, with low morbidity.

An alternative treatment strategy to intensive exogenous insulin therapy is transplantation of glucose-responsive insulin-producing human pancreatic tissue. The first transplant with pancreatic tissue was performed over a century ago (Williams 1894) but can now consist of either a whole pancreas transplant or isolated pancreatic islets. The first whole pancreas transplant for T1DM was performed in 1966 (Kelly, Lillehei et al. 1967) and is currently very successful, achieving >80% insulin independence (defined as euglycaemia without insulin for 14 or more consecutive days; (Close, Alejandro et al. 2007) one year post-transplant (Gruessner and Sutherland 2005). The 2007 annual report of the Scientific Registry of Transplant Recipients (SRTR) shows an 80% pancreas transplant graft survival at one year (for pancreas transplant alone) and 27% at 10 years post-transplant (SRTR 2007). Although pancreas transplantation is able to reverse diabetic complications (Navarro, Sutherland et al. 1997; Fioretto, Steffes et al. 1998; Larsen, Colling et al. 2004), its success comes with the morbidity of major surgery and a life-long requirement for immunosuppression. Both these obstacles preclude its widespread application to many patients, including children (Hathout, Lakey et al. 2003), making it a less than ideal treatment for T1DM.

Figure 4 Islet transplantation

Fig 4 (a)

Fig 4 (b)

Fig 4 (c)

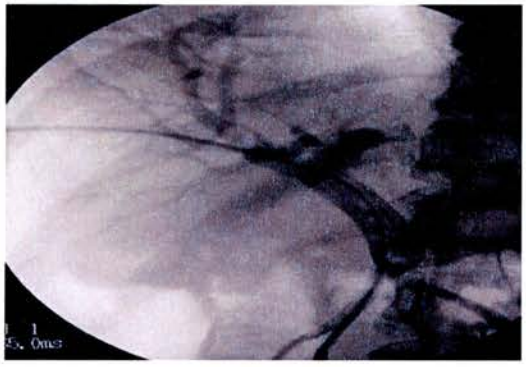


Fig 4 Islet transplantation is a minimally-invasive treatment, as shown in the photographs above. Fig 4 (a) shows the islet infusion being transplanted into a recipient. Fig 4 (b) shows the percutaneous portal venous catheter, through which the islet transplant is infused into the recipient. The infusion lasts approximately 10 to 15 minutes and is performed under antibiotic and heparin cover. The portal venous pressure is monitored throughout. Fig 4 (c) shows the radiologically-guided placement of the portal venous catheter, via a percutaneous transhepatic approach.

Islet transplantation, on the over hand, is a minimally-invasive alternative with low procedural morbidity (Frank, Barker et al. 2005). The technique involves isolating islets from deceased donor pancreatic tissue, using collagenase digestion and density gradient purification. These islets are then injected into the recipient liver, usually via ultrasound–guided percutaneous cannulation of the portal vein (see Figure 4). Since the introduction of the "Edmonton protocol" in 2000, this has achieved considerable success in treating selected patients, with >80% independent of exogenous insulin at 1 year post-transplantation (Shapiro, Lakey et al. 2000; Nanji and Shapiro 2006). Furthermore, successfully transplanted islets normalise serum HbA1C (Ryan, Lakey et al. 2002; Ryan, Paty et al. 2005) and secrete insulin in their characteristic physiological pulsatile manner (Porksen, Munn et al. 1994; Rorsman and Renstrom 2003) . The initial excitement of the 1 year follow-up data in Edmonton was tempered by 5 year follow-up data showing that the majority of recipients required a second islet transplant to achieve their insulin independence and only 10% remained insulin independent at 5 years post-transplant (Ryan, Paty et al. 2005). The average duration of insulin independence lasted only 15 months. Some noteworthy side effects of the immunosuppression and transplant were also reported, such as mouth ulcers, diarrhoea, anaemia, ovarian cysts, bleeding and branch portal vein thrombosis. However, longitudinal studies do confirm that true restoration of euglycaemia can be achieved, with reversal of diabetic hyperglycaemic complications (Fiorina, Folli et al. 2003; Lee, Barshes et al. 2005; Lee, Barshes et al. 2006). Islet transplantation also improves glycaemic lability, particularly the prevention of severe hypoglycaemic episodes requiring the assistance of another person and the sometimes very debilitating "hypoglycaemic unawareness" phenomenon, where the patient loses their ability to detect hypoglycaemia and may experience unexpected episodes of loss of

consciousness in some cases (Close, Alejandro et al. 2007). Prevention against severe hypoglycaemia and disease lability were enjoyed by the majority of recipients, including those not achieving or maintaining insulin–independence. Such cellular grafts also have the additional benefits of being suitable for tolerance induction and immunomodulation (Purrello and Pipeleers 1995; Coulombe and Gill 2004). However, islet transplantation does not completely restore the complex regulation of *in vivo* insulin secretion, as denervation of the islet grafts affects gustatory cephalic-phase insulin secretion (Berthoud, Trimble et al. 1980). In addition, beta-cell regulation from other cells within the islet may be affected. Although somatostatin-mediated beta-cell regulation is maintained, counter-regulation of hypoglycaemia by glucagon is impaired (Gupta, Wahoff et al. 1997; Kendall, Teuscher et al. 1997; Paty, Ryan et al. 2002; Rickels, Schutta et al. 2005; Rickels, Schutta et al. 2007).

The Collaborative Islet Transplant Registry (CITR) collects data from islet transplants in North America, and selected centres in Europe and Australia. Each year, the CITR provides a comprehensive overview of the cumulative data from 1999. In 2008, they reported that 72% of islet transplant recipients achieved insulin independence overall, whilst the insulin requirements of the remainder decreased substantially (CITR 2008). However, insulin independence decreased over time (see Figure 5). 71% of those achieving insulin independence remained so at 1 year post-transplant and 52% after 2 years. 11% received a further islet transplant 30 days after their first, and 65% within a year of their first transplant. Importantly, hypoglycaemic unawareness was markedly improved following transplantation and the only participants who experienced a severe hypoglycaemic attack were on insulin at the time.

Therefore, islet transplantation may be an excellent treatment for T1DM, as it is efficacious (normalises HbA1C, has endogenous insulin secretion kinetics, reverses diabetic complications) and safe (low procedural morbidity, has potential for use in children, predicted to have low long-term complications).

Figure 5 CITR 2008 report: persistence of insulin independence and graft function

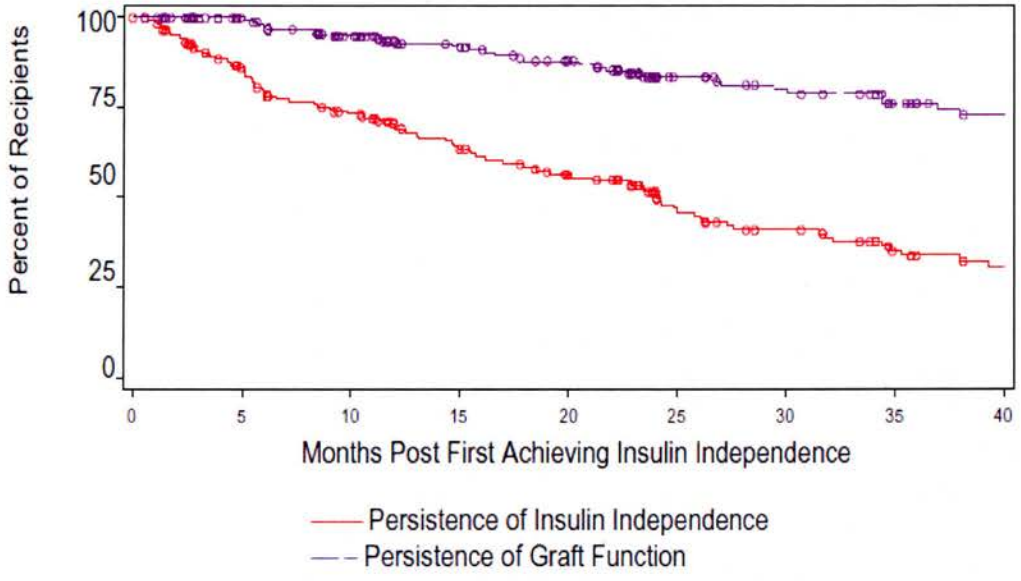


Fig 5 Persistence of insulin independence and graft function in islet alone recipients achieving insulin independence (from the CITR 2008 annual report). Over time there is a decrease in the sustainability of insulin independence. For islet alone recipients who ever achieved insulin independence, 71% had retained this status one year after achieving it and this decreases to 52% at two years. Similarly, graft function is lost over time but persists in a higher percentage of recipients compared to insulin independence.

1.2 Ongoing challenges for islet transplantation

Although intra-portal islet injection enjoys low procedural morbidity, its long-term safety is still unknown. This is highlighted by reports of focal liver steatosis in humans post-islet transplantation (Maffi, Angeli et al. 2005), with rat model studies suggesting a risk of liver cell proliferation (De Paepe, Keymeulen et al. 1995) and a potential for liver tumour formation (Dombrowski 2000). Furthermore, the potential use of islet transplantation to treat children with T1DM is currently hindered by a worldwide donor pancreas shortage and the risks associated with potential life-long immunosuppression. The islet requirement for insulin independence is very high, compounding the problem of donor pancreas shortages. Approximately 850,000 islets (11,000 islet equivalents / kg body weight) are currently required for successful outcomes with the Edmonton protocol. Interestingly, it is estimated that 70% of patients receiving islet autotransplantation after total pancreatectomy can achieve insulin independence with only 300,000 islets (Pyzdrowski, Kendall et al. 1992). Although advances in islet isolation techniques and less toxic immunosuppression may reduce the number of islets required to control glycaemia (Rood, Bottino et al. 2006), islet transplantation is currently restricted to a limited number of adult T1DM patients, in whom the morbidity of the disease and its treatment outweigh the risks of transplantation and lifelong immunosuppression. Since these risks currently outweigh the benefits of islet transplantation in children, they are not routinely eligible to receive islet allograft transplantation. In order to realise its full potential, however, islet transplantation needs to be made widely available to all T1DM patients, including children, prior to the onset of complications (Hathout, Lakey et al. 2003).

At present, recipients of islet transplants receive generalised immunosuppression to prevent allograft rejection and recurrent autoimmune islet destruction (Coulombe and Gill 2004; Noguchi, Matsumoto et al. 2006). Prior to the Edmonton Protocol in 2000, this would consist of protocols adapted from solid organ transplantation, consisting of azathioprine, cyclosporine and corticosteroids. However, fewer than 10% of patients were able to achieve insulin independence under these regimes. The Edmonton Protocol dramatically transformed clinical outcomes in islet transplantation through the introduction of a more potent, corticosteroid-free and less diabetogenic immunosuppressive regimen consisting of sirolimus, low-dose tacrolimus, and induction daclizumab (anti-interleukin-2 receptor antibody). In 2008, the CITR reported that 59% of recipients overall had received this protocol with their first islet transplant, but over two thirds of all islet cell transplants have involved different immunosuppression regimens during 2006 and 2007 (CITR 2008). Therapies used in recent years include Alemtuzumab, Anti-thymocyte globulin, Etanercept, Efalizumab, and Methylprednisolone. Mycophenolate mofetil (MMF) has been used at times in place of Sirolimus.

The limited long-term success of the Edmonton protocol may have been due to homeostatic expansion of an autoreactive $CD8^+$ T-cell population with that regimen (Monti, Scirpoli et al. 2008). MMF is gaining popularity amongst islet transplant immunosuppression protocols, following the discovery that it selectively inhibits T- and B-cell proliferation by suppressing *de novo* purine synthesis (Van Belle and von Herrath 2008). Therefore, expansion of the autoreactive T-cell population may be reduced with MMF, which may have implications for graft survival.

While insulin independence rates under modern immunosuppression protocols have been highly successful, patients must be maintained on lifelong immunosuppression, with its associated risks of toxicity, infertility, increased susceptibility to infection, risks of malignancy and post-transplant lymphoma (Nanji and Shapiro 2004; Burra, Buda et al. 2006).

Research continues into less toxic immunosuppression agents and future strategies for immunosuppression-free anti-rejection therapy, such as immune *tolerance induction* (Lohr, Knoechel et al. 2005; Delis, Burke et al. 2006) and *immunoisolation* devices (Klomp, Ronel et al. 1979). Tolerance induction shows a great deal of promise through techniques to block T-cell surface co-stimulatory molecules (Adams, Shirasugi et al. 2002), or through T-cell depletion (Calne, Moffatt et al. 2000), or possibly through donor-specific bone marrow transplantation (Delis, Burke et al. 2006). However, at present, these techniques are not yet efficacious or in widespread use and require adjunctive non-specific immunosuppression. Immunoisolation devices involve encapsulation of the donor islets to prevent interaction with host immune cells, but have so far experienced limitations from material bioincompatibility, islet hypoxia and failure to prevent all host immune pathways (Gray 2001; Kobayashi, Harb et al. 2006). However, this strategy ought to reduce immunosuppression requirements through prevention of the direct T-cell response. Following reports of prolonged graft function and T1DM reversal following human allotransplantation (Soon-Shiong, Heintz et al. 1994; Calafiore, Basta et al. 2006), phase I / II human trials are currently underway to assess the safety of encapsulated islets (eg: AmCyte). Research has also begun into macroencapsulation of donor islets with 3-D cell sheets

to form an immunoisolated "Bioartificial Pancreas" (Kobayashi 2008; Lee, Nishimura et al. 2008).

Even if the human cadaveric pancreatic donor shortage was overcome, it is estimated that this will still only meet 0.5% of the T1DM need (Hirshberg, Rother et al. 2003). Therefore, an alternative source of islets is still required. *Xenogeneic pancreases* offer a potentially unlimited source of islet tissue for transplantation (Trucco, Casu et al. 2007). However, xenografts are more immunogenic than human allografts, through complement-mediated hyperacute rejection, which results in marked early graft loss (Badet, Titus et al. 2001). Methods are under investigation to try and modulate this immune response, including co-transplantation with immunomodulatory cells such as Sertoli cells (Valdes-Gonzalez, Dorantes et al. 2005), co-stimulation blockade to prevent T-cell expansion and / or depletion (Hering, Wijkstrom et al. 2006), using human peptide expressing xenogeneic cells (Omori, Nishida et al. 2006), encapsulation techniques (Dufrane, Goebbels et al. 2006), or a combination of these approaches. These methods have experienced initial success in animal-to-animal models and initial animal-to-human transplantation experiments. However, they are still under investigation and further research into concerns over possible zoonotic infection transmission need to be addressed. For example, the infection risk to humans from "porcine endogenous retrovirus" (PERV) associated to leukaemia has been extensively debated and investigated, and until this issue is resolved it may be questionable to promote the widespread xenotransplantation of porcine islets (references within Limbert 2008). Although the concerns for disease potential from PERVs are easing, there is a paucity of evidence over the safety of this approach in humans and small-scale, well designed, carefully controlled clinical trials may allow a

better appreciation of the risks (Louz, Bergmans et al. 2008). The University of Minnesota (together with the Spring Point Project) plan to begin a clinical trial of porcine islet xenotransplantation into humans in 2009.

This failure to adequately resolve the problem of donor tissue paucity for islet transplantation has generated considerable worldwide interest in the potential that human pluripotent cells may hold in resolving this problem. Such cells may also avoid, or reduce, the need for immunosuppression currently required for allograft transplantation, thus improving the prospect for use in children with T1DM (see Chapter 1, section 1.4: *Potential pluripotent cell-based therapies for islet neogenesis*). In order to elucidate possible mechanisms of generating beta cells / islets from pluripotent cells, it is important to first consider normal pancreatic endocrine development.

1.3 Pancreatic organogenesis

Pancreatic organogenesis consists of three phases: *primary*, *secondary* and *tertiary* transitions. These are summarised in the following sub-sections and, for consistency, embryonic days are given in terms of murine gestation, which mirror the temporal relationships of avian and human pancreatic organogenesis (Streeter 1942; Streeter 1948; Park, Chae et al. 1992; Kim, Hebrok et al. 1997):

Primary transition (E8.5-E13)

This phase is characterised by the following events: pancreas specification, bud formation, "protodifferentiation" of pancreatic progenitors, epithelial branching and ventral bud rotation. *Pancreas specification* (E8.5) occurs in the pre-pancreatic

posterior foregut endoderm, through expression of both *Pancreas duodenum homeobox-1* (*Pdx-1*) and *Pancreas transcription factor 1a* (*Ptf1a*) in all progenitor cells contributing to lineages of both endocrine and exocrine pancreatic development (Gu, Dubauskaite et al. 2002; Kawaguchi, Cooper et al. 2002). Both of these transcription factors are required for pancreatic specification, whilst neither in isolation is sufficient (Afelik, Chen et al. 2006). *Pdx-1* expressing progenitors give rise to all cells of the pancreas (Gu, Brown et al. 2003) and lack of *Pdx-1* expression results in pancreatic agenesis (Jonsson, Carlsson et al. 1994). *Ptf1a* deficient mice show no pancreatic acinar cells and abnormal endocrine cell differentiation (Krapp, Knofler et al. 1998).

Upstream regulation of pancreatic specification is incompletely understood, but intrinsic factors regulating *Pdx-1* expression are likely to be involved. These include *forkhead box 2* (*Foxa2*), also known as *hepatocyte nuclear factor 3β* (*Hnf3β*) (Ang, Wierda et al. 1993; Cockell, Stolarczyk et al. 1995; Lee, Sund et al. 2005); (*sex determining region Y)-box* 17 (*Sox17*) (Kanai-Azuma, Kanai et al. 2002); *one cut domain 1* (*Onecut1*) (Jacquemin, Pierreux et al. 2003); *T-cell specific transcription factor 2* (*Tcf2*) (Haumaitre, Barbacci et al. 2005). Extrinsic inductive signalling responsible for initiation and maintenance of the intrinsic system includes *retinoic acid* (*RA*), *fibroblast growth factors* (*FGFs*), *transforming growth factor-β* (*TGFβ*) family proteins (such as *bone morphogenetic proteins* and *nodal / activins*). This extrinsic signalling is provided by posterior mesoderm (Stafford, Hornbruch et al. 2004), lateral plate mesoderm (Kumar, Jordan et al. 2003), notochord (Hebrok, Kim et al. 1998) and aortic endothelium (Jacquemin, Yoshitomi et al. 2006).

Pancreatic bud formation follows the onset of specification, with thickening and invagination of the specified endoderm into the surrounding mesenchyme, forming a *dorsal pancreatic bud* (E9.5) and *ventral pancreatic bud* (E10.5). Concurrently, glucagon-expressing cells (E9.5) and then insulin-expressing cells (E10.5) appear (Beaupain and Dieterlen-Lievre 1974; Dieterlen-Lievre and Beaupain 1974; Gittes and Rutter 1992; Kim, Hebrok et al. 1997). These protodifferentiated cells express sub-physiological amounts of hormone and have a limited capacity for mitosis (Pictet, Clark et al. 1972). These first hormone-expressing cells are not precursors of the definitive alpha- and beta- cells, which instead are derived from *neurogenin-3* (*Ngn3*) expressing endocrine precursors during secondary transition (Gu, Dubauskaite et al. 2002). It is therefore important to consider this important phenotypic difference between protodifferentiated hormone-expressing cells and definitive islet cell precursors when investigating potential pluripotent cell differentiation strategies.

Pancreatic epithelial branching begins at E11.5 and generates a primitive ductal network. The extracellular proteins *laminin* (Crisera, Kadison et al. 2000) and *collagens* (Hisaoka, Haratake et al. 1993) are required for pancreatic branching, which is controlled through mesenchyme-epithelial extrinsic signalling via *FGF10* (Bhushan, Itoh et al. 2001), *Hepatocyte Growth Factor* (Brinkmann, Foroutan et al. 1995), *Epidermal Growth Factor* (Miettinen, Huotari et al. 2000), *Indian Hedgehog* (Hebrok, Kim et al. 2000), *Activin* and *Follistatin* (Ball and Risbridger 2001).

Ventral pancreatic bud rotation occurs from E12.5, rotating behind the duodenum to reach a position adjacent to the dorsal bud (Slack 1995). The buds then fuse, possibly under the control of *hedgehog* and *TGFβ* signalling (Hebrok, Kim et al. 2000).

Secondary transition (E13.5-E16)

This phase achieves specification and determination of the endocrine and exocrine pancreatic cell lineages. Endocrine cell specification and differentiation is controlled by *neurogenin 3* (*Ngn3*), which is induced in epithelial cells from E9 by *Tcf1* (*Hnf1α*), *Foxa2* (*Hnf3β*) and *Onecut* (*Hnf6*) (Ang and Rossant 1994; Weinstein, Ruiz i Altaba et al. 1994; Pontoglio, Sreenan et al. 1998; Jacquemin, Durviaux et al. 2000; Lee, Smith et al. 2001). Interestingly, *Ngn3* is considered the key endocrine specification regulator, as it is both necessary and sufficient for all endocrine differentiation to occur (Gradwohl, Dierich et al. 2000; Gasa, Mrejen et al. 2004). Its expression is augmented during secondary transition, owing to a rapid endocrine precursor expansion, but then declines from E17.5 onwards to be absent at birth (Gradwohl, Dierich et al. 2000; Schwitzgebel, Scheel et al. 2000). Its expression is regulated through *Notch*- mediated lateral inhibition (Apelqvist, Li et al. 1999). Pro-endocrine *Ngn3* actually induces expression of *Notch* ligands, which in turn inhibit endocrine differentiation in adjacent precursors, leading to the initial scattered endocrine cell arrangement (Fujikura, Hosoda et al. 2006). Furthermore, pancreatic mesenchyme represses *Ngn3* expression through *Notch* signalling, but also produces *fibroblast growth factor 10* (*FGF10*) to maintain the *Notch* activation, and induces *hairy and enhancer of split 1* (*Hes1*), which favours precursor cell proliferation over endocrine differentiation (Hart, Papadopoulou et al. 2003; Miralles, Lamotte et al. 2006).

Ngn3 induces expression of a programme of three known transcription factors that are required for pancreatic endocrine differentiation; *NeuroD1* (Huang, Liu et al. 2000), *insulinoma-associated 1* (*IA1*) (Gierl, Karoulias et al. 2006; Mellitzer, Bonne et al. 2006), and *islet 1* (*Isl-1*) (Ahlgren, Pfaff et al. 1997). Once committed to an endocrine

fate, *paired homeobox 4* (*Pax4*) and *aristaless related homeobox* (*ARX*) determine islet cell lineages; *Pax4* differentiating towards beta- and delta-cell fates and *ARX* to alpha- and epsilon-cell fates (Collombat, Mansouri et al. 2003; Heller, Jenny et al. 2005). The individual islet cell lineages then proceed to terminal differentiation along independent and distinct transcriptional pathways, summarised below and reviewed (with references) within Bonal and Herrera 2007:

Beta cells: *Pax4, Pax6, NK homeobox 2.2* (*Nkx2.2*), *Nkx6.1, v-maf musculoaponeurotic fibrosarcoma oncogene A* (*MAFA*), *H2.0-like homeobox b9* (*Hlxb9*), *Pdx-1*

Alpha cells: *ARX, Pax6, Nkx2.2, MAFB, brain-specific homeobox 4* (*Brn4*), *Foxa2*

Delta cells: *Pax4, Pax6* (Collombat et al. 2003; 2005)

Pancreatic Polypeptide cells: *Nkx2.2* (Sussel, Kalamaras et al. 1998)

Epsilon cells: not yet described, but repressed by *Pax4* and *Nkx2.2* (Chao, Loomis et al. 2007; Wang, Elghazi et al. 2008)

The scattered epithelial endocrine precursors also migrate during secondary transition to form irregular islet cell clusters (isletogenesis). This occurs under *FGF2* induction (Hardikar, Marcus-Samuels et al. 2003), whilst cell migration is permitted via degradation of the extracellular matrix (ECM) via *TGFβ* (Miralles, Battelino et al. 1998).

Exocrine differentiation of acinar and ductal tissue from *Ptf1a* expressing precursors occurs during secondary transition, under as yet unknown extrinsic mesenchymal

signals. This is shown by absent exocrine differentiation in mice lacking dorsal pancreatic mesenchyme (Ahlgren, Pfaff et al. 1997).

Tertiary transition (E19 - 3 weeks after birth)

The existing primitive islet cell clusters now undergo extensive remodelling into adult islet morphology, through coordinated proliferation, apoptosis and islet neogenesis. The exocrine tissue undergoes extensive growth, leading to acinar cell condensation into discrete adult pancreatic acinae (Jensen 2004). Both exocrine and endocrine cells undergo final differentiation during early neonatal life into physiologically functional adult pancreatic tissue. Until this final maturation step, islet cells are poorly responsive to a glucose stimulus (Hughes 1994). This marks the final step of normal pancreatic organogenesis.

1.4 Potential pluripotent cell-based therapies for islet neogenesis

When considering islet regeneration, it is interesting to consider why we should wish to regenerate islets, rather than purely beta-cells? The benefits of islet replacement over beta-cell replacement include: enhanced glucose-responsive insulin secretion through autocrine stimulation between adherent beta-cells (Aspinwall, Lakey et al. 1999; Hauge-Evans, Squires et al. 1999); advantageous insulin, glucagon and somatostatin secretion kinetics through paracrine signalling between beta- and non-beta cells within the islet (Brunicardi, Kleinman et al. 1994; Kleinman, Ohning et al. 1994; Kanno, Gopel et al. 2002; Ishihara, Maechler et al. 2003; Hope, Tran et al. 2004; MacDonald and Rorsman 2006); superior graft function when beta-cells are co-transplanted with non-beta cells (Keymeulen, Korbutt et al. 1996); islet regeneration and insulin secretion are both supported through maintenance of the neural network

and vascular endothelium present with intact islets (Porksen, Munn et al. 1994; Hess, Li et al. 2003; Mathews, Hanson et al. 2004). Therefore, there are distinct advantages to achieving regeneration of whole islets rather than pure beta-cells. Most research in the literature has so far primarily focussed on trying to achieve beta-cell regeneration, although there has been a report of *in vitro* single cell precursor expansion into cell clusters comprising several islet cell types, including alpha, beta and delta cells (Seaberg, Smukler et al. 2004).

The search for a renewable and expandable islet source has produced considerable interest in the "stem cell" field, which holds a great deal of promise for the future treatment of Diabetes Mellitus (Shamblott and Clark 2004; Bonner-Weir and Weir 2005). Stem cells can be defined as undifferentiated cells with continued proliferative capacity, yielding both undifferentiated daughter cells (self-renewal) and differentiating daughter cells of more than one cell type (Shostak 2006). Three main types of stem cells exist: *embryonic stem cells* (ES cells), *foetal stem cells* and *adult stem cells* (Bonner-Weir and Weir 2005). A stem cell strategy would attempt to produce new islets (islet neogenesis) through recapitulation of pancreatic organogenesis, instructing pluripotent cells to differentiate into a safe and functional islet phenotype via lineage-specific transcriptional signalling. However, many questions still remain, including: Which is the optimal pluripotent stem cell type to use for this purpose? How can we successfully produce fully functional islets (or beta-cells) from such a source? What are the exact molecular mechanisms controlling normal pancreatic organogenesis? Does a suitable adult stem cell (or an islet progenitor) exist and where is it located (pancreatic or extra-pancreatic)?

1.4.1 Embryonic stem cells

Pancreatic islet stem cell research has so far largely focussed on embryonic stem (ES) cell strategies to try and differentiate cells from the inner cell mass of pre-implantation blastocyst embryos (Evans and Kaufman 1981; Martin 1981; Thomson, Itskovitz-Eldor et al. 1998) down a pancreatic endocrine lineage. ES and ES-derived cells express low levels of major histo-compatibility complex (MHC) class I and lack MHC class II protein expression (Drukker, Katz et al. 2002), making these cells "immune privileged", which may afford some protection against host-versus-graft disease (HVGD) following allotransplantation (Fandrich, Lin et al. 2002; Hori, Ng et al. 2003; Drukker 2004; Drukker and Benvenisty 2004; Li, Baroja et al. 2004; Drukker, Katchman et al. 2006). ES cells hold a great deal of natural potential to undergo islet differentiation. Although a rare event (<0.1%), spontaneous differentiation into insulin-producing cells *in vitro* has been reported in mouse (Houard, Rousseau et al. 2003) and human (Assady, Maor et al. 2001) ES cell lines. Therefore, directed differentiation protocols have been developed trying to recapitulate normal *in vivo* islet developmental cues. These include co-culturing ES cells with differentiating foetal islet tissue (Brolen, Heins et al. 2005); nestin selection of putative ES-derived islet precursor cells (Lumelsky, Blondel et al. 2001); targeted differentiation of ES cells into endoderm (D'Amour, Agulnick et al. 2005) then islet precursors (Hebrok, Kim et al. 1998) through culture manipulations; targeted differentiation of ES cells through forced expression of key pancreatic genes, such as *Pdx-1* (Treff, Vincent et al. 2006); maturation of islet precursors into functional surrogate beta-cells (Soria, Roche et al. 2000). However, ES cell studies have so far failed to successfully achieve differentiation into adequate islet beta-cell tissue, which is safe, fully-functional and sufficiently glucose-responsive (Heit and Kim 2004;

Stainier 2006). Also, it is important to note that the use of human ES cells has encountered ethical and religious controversies over the worth and moral right of developing embryos (Oakley 2002; Copland 2004; McConnaha 2005). ES cell research is therefore subject to strict governmental regulation and is even banned in some countries (Gruss 2003; Halme and Kessler 2006). Novel techniques for ES cell harvest, which do not impair the development of the embryo, have recently been developed, such as single blastomere derivation (Chung, Klimanskaya et al. 2006; Chung, Klimanskaya et al. 2008). These may reduce ethical barriers to the use of human ES cells (Jaenisch and Meissner 2006; Taupin 2006). However, safety concerns remain over the risk of *in vivo* teratoma formation in ES-derived tissue, from uncontrolled differentiation into alternative phenotypes (Thomson, Itskovitz-Eldor et al. 1998; Amit, Carpenter et al. 2000). Rapidly dividing undifferentiated ES cell proliferation may predispose to genomic instability and neoplastic transformation. In addition, ES-derived cells may exhibit phenotypic instability and dedifferentiate, resulting in a loss of the desired phenotype, with a return to a more undifferentiated and potentially pro-teratogenic state (Draper, Smith et al. 2004; Inzunza, Sahlen et al. 2004; Fujikawa, Oh et al. 2005). These safety concerns must first be resolved before ES-derived cells become a suitable candidate tissue for cellular transplantation in humans.

1.4.2 Foetal stem cells

Foetal stem cells refer to pluripotent cells derived from the foetus. Not only might these cells provide a useful pluripotent cell source for islet transplantation, but the study of foetal pancreatic pluripotent cells is also useful in understanding the process

of islet differentiation. Foetal cells are a potentially attractive cell source, as they also enjoy "immune privileged" status, making them less susceptible to rejection (Bai, Maedler et al. 2004). Furthermore, islet precursor proliferation and differentiation occurring *in vivo*, following foetal tissue transplantation, reduces the minimum graft volume needed to restore the beta-cell mass (Hammerman 2004). Foetal islets also appear resistant to factors impairing engraftment, such as hypoxia-induced apoptosis (Emamaullee, Shapiro et al. 2006). Foetal pluripotent cells capable of *in vitro* differentiation into insulin-producing cells and capable of reversing diabetes in experimental mouse models have been acquired from: the foetal pancreas itself (Wu, Jagir et al. 2004), foetal gut (Suzuki, Nakauchi et al. 2003), foetal liver (Zalzman, Gupta et al. 2003) and foetal bone-marrow (Ai, Todorov et al. 2007). Alternatively, foetal beta-cell precursors themselves can be transplanted and then undergo *in vivo* proliferation and differentiation into a functional beta-cell mass (Beattie, Lopez et al. 1999). Experimental transplantation of foetal pancreatic islets has also been attempted in humans, using human (Dordevic, Lalic et al. 1995) or porcine (Valdes-Gonzalez, Dorantes et al. 2005) foetal islet tissue. However, recipients rarely achieve insulin-independence, although they show demonstrable improvements in glycaemic control (Djordjevic, Lalic et al. 2004; Valdes-Gonzalez, Dorantes et al. 2005) and diabetic complications (Farkas, Fulop et al. 1997). Widespread clinical application may be hindered by limited availability and ethical constraints of using human foetal islet tissue (Mandel 1999). The immunobiology of foetal islet tissue transplantation also remains very complex, with incomplete immune tolerance of foetal tissue (Bennet, Bjorkland et al. 2000; Solomon, Kuziel et al. 2004), and recurrence of autoimmunity following foetal islet transplantation in humans (Brooks-Worrell, Peterson et al. 2000). If xenotransplantation was used to address the lack of foetal tissue availability,

using foetal porcine tissue for example, then the immunological and safety concerns of this would also need to be carefully considered (Mandel 1999).

1.4.3 Adult stem cells

These concerns have generated interest in the adult stem cell field, using pancreatic or extra-pancreatic adult stem cells, or islet progenitors, located within differentiated tissues of the foetus, child or adult to derive the islet tissue (Weissman 2000). Adult stem cells have a more restricted developmental potential than ES cells and this may limit the breadth of phenotypes they can adopt. Despite this, pluripotent cells can adopt cell fates beyond their normal developmental potential under favourable circumstances, such as bone-marrow mesenchymal stem cells adopting a nerve cell fate, for example (Sugaya 2003). Furthermore, adult stem cells may be less tumorigenic (and less likely to undergo teratoma formation) than ES cell-derived tissue (Grompe 2002). This less contentious pluripotent cell type may also overcome the ethical issues surrounding ES cell therapy and make such a cell-based therapy more applicable for human use. Deriving the adult stem cells from the diabetic patient may also reduce generalised immunosuppression needs currently required with allografts (Noguchi, Matsumoto et al. 2006) with its associated risks, which currently generally excludes children from receiving islet transplant therapy (Hathout, Lakey et al. 2003). It is currently unknown whether the human diabetic patient's own immune system would tolerate replacement autografted beta-cells derived from adult stem cells (Burns, Persaud et al. 2004; Weir 2004), or whether the transplanted beta-cells would undergo the same targeted autoimmune-mediated destruction as their predecessors (Soeldner, Tuttleman et al. 1985; Atkinson and Maclaren 1994;

Atkinson and Eisenbarth 2001). However, there has been a recent report that human adult stem cells are capable of expressing high levels of MHC class I proteins, and so may be rejected on transplantation unless modified (Kim, Kim et al. 2005). Therefore, a concomitant immunomodulatory strategy may be required to protect adult stem cell derived islets from attack.

A naturally occurring adult islet stem / progenitor cell has remained elusive and even its existence remains controversial in diabetes research. Beta-cell turnover does occur naturally, although more slowly in humans than in mice (Bouwens and Pipeleers 1998; Butler, Janson et al. 2003; Bouwens and Rooman 2005; Dor 2006). Formation of new beta-cells *in vivo* may occur via *neogenesis* through differentiation of a beta-cell progenitor / pluripotent stem cell (Bonner-Weir, Toschi et al. 2004; Bonner-Weir and Sharma 2006) and / or via *replication* of pre-existing fully differentiated beta-cells (Dor, Brown et al. 2004; Dor and Melton 2004; Dor 2006). *In vivo* genetic lineage tracing studies indicate that in healthy adult mice, the beta-cell population is maintained during adult life through existing beta-cell replication rather than an adult stem cell population (Dor, Brown et al. 2004). Detectable C-peptide levels and residual beta-cells do exist in 11 to 40% of patients with longstanding T1DM (Rother and Harlan 2004). Therefore, beta-cell mass regeneration through replication may offer a potential future therapeutic strategy for some patients, if the autoimmune process was ameliorated and the residual beta-cell mass could be expanded sufficiently. The replication theory has recently been demonstrated in partially pancreatectomised adult mice, where new beta-cells formed through replication of existing beta-cells (Dor, Brown et al. 2004; Ackermann Misfeldt, Costa et al. 2008). Another study reports partial recovery of endogenous islet beta-cell function in

streptozotocin-induced diabetic mice, through a combination of beta-cell proliferation and hypertrophy, following removal of an 120 day syngeneic islet transplant (Yin, Tao et al. 2006). Pancreatic islet beta-cell regeneration (and transient liver cell transdifferentiation into insulin-producing cells) has also been reported to restore normoglycaemia when recombinant *Pdx-1* intra-peritoneal injections are administered to streptozotocin-induced diabetic mice (Koya, Lu et al. 2008). However, this remarkable proof-of-principle report faces many obstacles to clinical translation (including safety concerns, degradation of the protein by serum proteases, toxicity to non-target organs etc.) and further studies are required. In addition, such a therapy may not be widely applicable to those T1DM patients with no residual beta-cells left to regenerate.

It is not known whether *in vivo* beta-cell mass regeneration may also occur through neogenesis from progenitor cells, or an adult stem cell population. An adult islet progenitor, or an adult pancreatic pluripotent cell, capable of deriving cells with phenotypic similarities to beta-cells have been identified in the pancreas itself (Cornelius, Tchernev et al. 1997; Linning, Tai et al. 2004; Lechner, Nolan et al. 2005), and within specific pancreatic tissues; such as exocrine / acinar tissue (Baeyens, De Breuck et al. 2005; Todorov, Omori et al. 2006; Okuno, Minami et al. 2007; Jetton, Everill et al. 2008), ductal tissue (Bonner-Weir, Taneja et al. 2000; Wang, Rosenberg et al. 2005; Xu, D'Hoker et al. 2008) or islet tissue (Zulewski, Abraham et al. 2001; Abraham, Kodama et al. 2004; Gershengorn, Hardikar et al. 2004; Eberhardt, Salmon et al. 2006). The recent report of insulin-producing cell production from facultative endocrine progenitors in the ductal lining of the adult mouse pancreas upon injury is particularly noteworthy (Xu, D'Hoker et al. 2008).

Taken together with Dor *et al*'s findings, this suggests that in the adult mouse the beta-cell population may be maintained by self-duplication during health, but that islet neogenesis via an islet progenitor or adult stem cell can be utilised at times of stress (Dor, Brown et al. 2004; Xu, D'Hoker et al. 2008). Despite these findings, an adult pluripotent cell within the pancreas which is capable of yielding tissue suitable for diabetes reversal in humans has not yet been successfully realised. If a ductal lining progenitor does exist in humans, it may be problematic expanding a sufficient number for diabetes reversal, or safely and readily accessing these cells for autotransplantation (but could theoretically be achievable through Endoscopic Retrograde Cholangio-Pancreatography).

This has led to research into potential alternative sites of adult islet stem / progenitor cells, or other differentiated cells which may be amenable to *transdifferentiation* or *dedifferentiation* strategies. Transdifferentiation refers to pluripotent cell maturation and differentiation which crosses the tissue-lineage boundaries of its normal developmental potential, or even the switching of a fully differentiated cell into a new phenotype (Slack and Tosh 2001; Tosh and Slack 2002). *Dedifferentiation*, on the other hand, refers to reversion to an immature intermediate precursor, which may precede the acquisition of a new phenotype. Interestingly, there have been recent reports where both mouse and human differentiated adult somatic cells have been induced into a pluripotent state by virus-mediated transfection with pluripotency genes, including *octamer 4* (*Oct4*), *Sox2*, *cellular myelocytomatosis oncogene* (*c-myc*) and *Kruppel-like factor 4* (*Klf4*) (Takahashi, Tanabe et al. 2007; Aoi, Yae et al. 2008; Bru, Clarke et al. 2008; Huangfu, Maehr et al. 2008). Some of the specific somatic cells used include human adult fibroblasts (Takahashi, Tanabe et al. 2007) and adult

mouse liver cells, stomach cells and terminally differentiated pancreatic beta-cells (Aoi, Yae et al. 2008; Stadtfeld, Brennand et al. 2008). Furthermore, these dedifferentiated induced human pluripotent cells had similar properties to human ES cells (in morphology, proliferation, surface antigens, telomerase activity, gene expression and epigenetic status) and were capable of differentiating into cells of all three germ layers (Takahashi, Tanabe et al. 2007; Stadtfeld, Brennand et al. 2008). There has been a recent report of generating insulin-secreting islet-like cell clusters from induced pluripotent cells derived from human skin fibroblasts (Tateishi, He et al. 2008). Again, these experiments used *Oct4*, *Sox2*, *c-myc* and *Klf4* to induce the human skin fibroblasts into pluripotency, before differentiating the cells down a differentiation protocol previously used to produce insulin-secreting islet-like clusters from human embryonic stem cells. The prospect of using differentiated adult somatic cells for islet neogenesis is certainly very exciting, as these cells are readily available from the patient and would overcome the ethical barriers to using ES cells. However, these are early reports showing proof-of-principle, but at extremely low frequencies, and little is still known about the mechanisms behind this process. Furthermore, the stability and safety of these "induced" cells would also need to be firmly established, in particular whether viral integration is associated with any genomic instability and whether inducing these cells into a more immature rapidly proliferating phenotype might predispose to neoplastic transformation?

Potential alternative sites of adult stem cells, progenitor cells, or cells which may be capable of transdifferentiating into insulin-producing cells (and also functionally tested, in the majority of cases, as capable of reversing chemically-induced diabetes) include; pancreatic exocrine tissue (Zhou, Brown et al. 2008), the pancreatic duct

(Bonner-Weir, Inada et al. 2008; Xu, D'Hoker et al. 2008), the gut (Cheung, Dayanandan et al. 2000; Fujita, Cheung et al. 2004; Lear, Jayanthi et al. 2004), the liver (Yang, Li et al. 2002; Nakajima-Nagata, Sakurai et al. 2004; Yamada, Terada et al. 2005; Tang, Cao et al. 2006; Koya, Lu et al. 2008), the salivary gland (Matsumoto, Okumura et al. 2007), the placenta (Battula, Bareiss et al. 2007), the amnion (Wei, Zhang et al. 2003), umbilical cord blood (Zhao, Wang et al. 2006; Sun, Roh et al. 2007) , nervous tissue (Hori, Gu et al. 2005), bone marrow (Ianus, Holz et al. 2003; Oh, Muzzonigro et al. 2004; Li, Zhang et al. 2007), peripheral blood (Ruhnke, Ungefroren et al. 2005), adipose tissue (Timper, Seboek et al. 2006; Lee, Han et al. 2008) and the spleen (Kodama, Kuhtreiber et al. 2003).

The recent report of *in vivo* reprogramming of differentiated pancreatic exocrine cells in adult mice into cells that closely resemble beta-cells has generated a great deal of interest (Zhou, Brown et al. 2008). This transdifferentiation was achieved *in vivo* by re-expressing a specific combination of three transcription factors (*Ngn3*, *Pdx-1* and *Mafa*) in the exocrine cells of the pancreas by adenoviral vector transfer. This combination of transcription factors resulted in the conversion of >20% of infected cells into insulin-expressing cells, which were first detected 3 days after injection. These induced cells were indistinguishable from islet beta-cells and this reprogramming effect appeared to be rather specific for pancreatic exocrine cells, as infected skeletal muscle or fibroblasts did not express insulin. Streptozotocin-induced diabetic mice also experienced a significant improvement in their hyperglycaemia following infection of their pancreas with the adenovirus (and the virus did not appear to spread to other organs).

Umbilical cord blood has also generated considerable interest as an accessible, abundant and ethical pluripotent cell source for cell-based therapies (Koblas, Harman et al. 2005; Greschat, Schira et al. 2008), which can be differentiated into insulin-producing cells (Sun, Roh et al. 2007) capable of restoring normoglycaemia in experimental models of diabetes (Zhao, Wang et al. 2006). However, the safety of such cells for future therapies has yet to be determined (Koblas, Harman et al. 2005).

The potential role of the human spleen as a future source of adult stem cells for islet transplantation is discussed and reviewed in the next chapter.

Chapter Two

The spleen as a potential tissue source for islet transplantation

2.1 Mesenchyme-to-Epithelial Transition

Epithelium and *mesenchyme* are two phenotypically distinct tissue types. Epithelium is characterised by tightly-packed sheets of cells adherent to one another (through tight junctions, gap junctions and adherent junctions) and to the underlying extracellular matrix (ECM) or basement membrane. Epithelium is typically non-migratory and has a recognisable architecture. Contrastingly, mesenchyme is comprised of loosely arranged cells, which rarely have cell-cell adhesions. Mesenchyme is migratory, by way of its pseudopodia, and adopts no specific recognisable architecture.

Despite these disparate phenotypes, cells do undergo transitions between epithelium and mesenchyme during the requirements of development or the nature of disease. For example, *epithelial-to-mesenchyme transition (EMT)* occurs during development in the process of gastrulation, when the epiblast cells ingress to form mesoderm and endoderm primary germ layers (Stern 2004). EMT is also seen in disease, where it is an important determinant of malignant transformation and tumour invasion (Grille, Bellacosa et al. 2003). Examples of *mesenchyme-to-epithelial transition (MET)* during development include somite formation from paraxial mesoderm (Nakaya, Kuroda et al. 2004) and kidney organogenesis (Davies and Fisher 2002). During kidney development, signals from the ureteric bud induce epithelialisation of condensed metanephric mesenchyme, leading to definitive nephron formation. It is interesting to note that the kidney was originally thought to be derived exclusively from epithelium, but now the role of MET in its organogenesis is firmly established (Davies and Fisher 2002; Denholm, Sudarsan et al. 2003).

In the search for a pluripotent cell source of replacement islets to reverse Diabetes Mellitus, it is important to understand the developmental origin of pancreatic islet cells. Previous fate mapping studies have revealed that cells of the endocrine and exocrine pancreas are derived exclusively from pancreatic endodermal epithelium (see Chapter One, section 1.3; *Pancreatic Organogenesis*), under the influence of signals from the lateral plate mesoderm (Percival and Slack 1999; Kumar, Jordan et al. 2003). These studies showed that embryonic pancreatic epithelium was competent to derive pancreatic islet, acinar and ductal tissue, finding no examples of a cellular contribution from the mesenchyme. This finding is supported by expression of regulatory genes required for pancreatic organogenesis by the foregut epithelium (Jensen 2004). However, recent studies have demonstrated novel ways of recapitulating beta-cell neogenesis, using alternative transcriptional networks (Servitja and Ferrer 2004). Several groups have now elucidated a specific role for transitions between mesenchymal and islet cell-types in the development and regeneration of islet tissue (Kodama, Kuhtreiber et al. 2003; Gershengorn, Hardikar et al. 2004; Atouf, Park et al. 2007; Chase, Ulloa-Montoya et al. 2007).

Previous findings from our own laboratory have shown that the developing foregut mesenchyme (from the developing stomach mesenchyme or pancreatic mesenchyme) is capable of differentiating into pancreatic endocrine and exocrine tissue when combined with pancreatic epithelium in a model of developing avian pancreatogenesis (Lear, Jayanthi et al. 2004; Teague, Jayanthi et al. 2005; Teague, Rowan-Hull et al. 2006; Teague, Rowan-Hull et al. 2007).

These findings of islet Mesenchyme-to-Epithelial Transition (iMET) are supported by observations from other groups: Firstly, the reported reversal of diabetes in adult Non-Obese Diabetic (NOD) mice when transplanted with adult donor mouse splenocytes (with attenuation of autoimmunity) by *in vivo* transdifferentiation of mesenchyme-derived splenocytes into epithelial beta-cells (Kodama, Kuhtreiber et al. 2003). Secondly, cellular gene expression of endogenous insulin has been observed in cultures of mesoderm and ectoderm of E7.5 gestation mice devoid of endoderm (Wells and Melton 2000). Thirdly, epithelia of renal nephrons are now known to differentiate via MET in the kidney, as previously mentioned (Davies, Perera et al. 1999; Denholm, Sudarsan et al. 2003), whilst epithelia of lymphatic vessels also undergo MET (Wilting, Papoutsi et al. 2001). Finally, the important mediator of renal MET, *Wilms tumour suppressor gene 1* (*Wt1*), is also expressed at the onset of pancreatogenesis by the presumptive dorsal pancreatic mesenchyme (Armstrong, Pritchard-Jones et al. 1993).

We were therefore now interested in investigating whether the developing spleen, itself a mesodermally-derived organ (Brendolan, Rosado et al. 2007), could also undergo MET to produce islets.

2.2 The spleen

The vertebrate spleen is a lymphoid organ that plays important roles in haematopoiesis, the generation of primary immune responses and filtration of blood (to remove aged or abnormal blood cells and remove intraerythrocytic inclusions) (van Rooijen, Claassen et al. 1989; Zapata and Cooper 1990; Chadburn 2000; Brendolan, Rosado et al. 2007). The spleen also stores a small fraction of the body's

red blood cells. These functions are performed by the red pulp of the spleen. The white pulp, on the other hand, is the lymphoid compartment of the spleen. This tissue releases neutrophils to counter infection and produces antibodies against invading pathogens. It also stores upto one third of the body's platelets and releases these to counter bleeding. Removal of the spleen can result in Overwhelming Post-Splenectomy Infection (OPSI), as the body can subsequently struggle to fight certain invading microorganisms (especially encapsulated bacteria) and it is usually necessary for patients undergoing splenectomy to receive immunisations prior to the procedure (currently Pneumococcus, Haemophilus Influenzae B and Neisseria Meningitidis vaccinations are given in clinical practice) plus long-term prophylactic antibiotics (Waldman, Rosenthal et al. 1977; Jirillo, Mastronardi et al. 2003; Okabayashi and Hanazaki 2008).

2.3 Developmental anatomy of the spleen

The development of the spleen has previously been described in chick embryos from E4 until after hatching using a number of cytological markers (Yassine, Fedecka-Bruner et al. 1989). This organ undergoes dramatic functional changes during ontogeny and switches from haematopoietic to immune function. The chick spleen rudiment first becomes recognisable at E3 as a slight bulge of the dorsal mesentery capping the dorsal pancreatic bud (Yassine, Fedecka-Bruner et al. 1989). The splenic and pancreatic mesoderm remain continuous until E5. At E6, as the duodenal loop grows out, the splenic bud separates from the pancreas and forms a loose network of mesenchymal cells. From E3 to E9 this loose mesenchymal network becomes progressively interspersed with basophilic cells. From E9 to E13, the basophilic cells are very abundant and erythropoiesis begins. The first reticular fibres become visible

in the chick embryonic spleen at E11. By E11.5, the splenic anlage is lined by a mesothelial sheath, which will form the prospective splenic capsule. From E13 to hatching, granulopoiesis is active, becoming the dominant process from E15, as erythropoiesis decreases. The first T-lymphocytes are observed in the spleen on E16 and the first B-lymphocytes on E18 (Hoffmann-Fezer, Rodt et al. 1977). For comparison, in the mouse embryo, free mononuclear cells are scattered throughout the splenic anlage at E15 and the spleen is prehaematopoietic (Sasaki and Matsumura 1988). Immature erythroid cells and small lymphocytes first appear in the mouse embryonic spleen at E16 and the spleen then becomes erythropoietic.

2.4 Gene expression during splenic development

The genetic control of splenic development is poorly understood, although recent studies in mutant mice have shown several genes are important for the spleen to develop normally. These include *T-cell leukaemia homeobox 1* (*Tlx-1*; previously known as *Hox-11*), *pre-B-cell leukaemia homeobox 1* (*Pbx1*), *podocyte-expressed 1* (*Pod*-1; also known as *capsulin* or *epicardin)*, *Wt1*, *Nkx2.3*, *bagpipe homeobox factor 1* (*Bapx1; also known as Nkx3.2*), *Nkx2.5.* However, the exact relationship between these genes remains undetermined.

2.4.1 Tlx-1 (Hox-11)

Tlx-1 (previously known as *Hox-11*) is a transcription factor encoded by a highly conserved homeobox gene whose expression is critical for the specification of splenic fate. It is expressed in various locations in the normally developing mouse embryo,

including the branchial arches, pharynx, heart, hindbrain, pinna, external auditory meatus and spleen (Raju, Tang et al. 1993; Roberts, Shutter et al. 1994; Dear, Colledge et al. 1995; Logan, Wingate et al. 1998). It is initially expressed at E8.5 in the developing muscle plates of branchial arches 1, 2 and 3 and subsequently in the motor nuclei that innervate them, cranial nerves V, VII, and IX respectively. *Hox-11* was also noted to be on in a single site within the abdomen (the splanchnic mesoderm) beginning at E11.5. Roberts *et al* first reported the role of this gene in splenic development (Roberts, Shutter et al. 1994). They found that *Hox-11* was expressed in a highly localised portion of the splanchnic mesoderm destined to form the spleen, beginning at E11.5. This tissue condensed into a small mass on the dorso-lateral margin of the future stomach and continued to express *Hox-11* as it proliferated to form the definitive spleen. It continued to be expressed in the developing spleen through E13.5 but was down-regulated thereafter (weaker expression at E14.5). Interestingly, they reported that expression of this gene was required for splenic genesis, as their *Hox-11* -/- mice had no spleen but otherwise appeared normal. In addition, many of the erythrocytes in these mice contained Howell-Jolly bodies, a finding consistent with asplenia.

However, Dear *et al* subsequently discovered that *Hox-11* is not required to *initiate* splenic development but is instead essential for the *survival* of splenic precursors during development (Dear, Colledge et al. 1995). They similarly observed that newborn *Hox-11* -/- mice exhibit asplenia. They found that splenic formation begins normally at E11.5 in *Hox-11* -/- mice mutant embryos, but the spleen anlage then underwent rapid and complete resorption between E12.5 and E13.5, showing molecular features of apoptosis, rather than inflammation or cell lysis. In *Hox-11* +/-

mice, sections of E18.5 spleen show *Hox-11* expression in the outer capsule of the spleen and the trabeculae, which together form the framework of the spleen. *Hox-11* was not seen in the erythroid precursors within the spleen. They explain the splenic atrophy observed in their *Hox-11* -/- mice by the inability of this splenic framework to survive. However, it was only splenic development that seemed affected by *Hox-11* null mutants, possibly via functional redundancy of related genes. Interestingly, Roberts *et al* also performed a Terminal Transferase Uridyl Nick End Labelling (TUNEL) assay (Gavrieli, Sherman et al. 1992) on their *Hox-11* -/- mice to see if the spleen actually atrophied, but serial sections at multiple time points from embryos revealed no increase in cell death within the dorsal mesogastrium at the site where the spleen would normally develop (Roberts, Sonder et al. 1995). They observed instead an increased stomach size, and possibly also the pancreas, in their *Hox-11* -/- mice suggesting that cells normally destined to form the spleen may adopt the fate of surrounding cells in the absence of *Hox-11*, rather than undergoing apoptosis

Logan *et al* studied *Tlx-1* (*Hox-11*) expression in the sensory nervous system of the chick embryo (Logan, Wingate et al. 1998). Expression was noted in similar regions to that previously reported in the mouse (hindbrain, heart, branchial arches etc) and prominent expression from stage 20 onwards in mesenchymal cells of the dorsal mesentery of the gut region, which corresponded to the splenic primordium.

Hox-11 expression is known to persist until birth in the mouse (Kanzler and Dear 2001) and there is evidence for continued expression of this early developmental marker in CD45 -ve cells of the adult human spleen (Kodama, Davis et al. 2005; Dieguez-Acuna, Gygi et al. 2007). However, to the best of our knowledge and

following a thorough literature search, *Tlx-1* (*Hox-11*) expression in the embryonic chick spleen has not yet been formally characterised during development (see Chapter Five).

2.4.2 Pbx-1

Absence of *Pbx-1* expression results in asplenia within mice embryos with 100% penetrance (Selleri, Depew et al. 2001). *Pbx-1* deficient mice died at E15 / E16 (Selleri, Depew et al. 2001; Brendolan, Ferretti et al. 2005) from severe hypoplasia or aplasia of multiple organs, including asplenia but also pancreatic morphogenesis and function (Kim, Selleri et al. 2002), amongst others. They additionally had widespread skeletal patterning defects. The exact reason for the asplenia in these mice is unclear but *Pbx-1* has been reported to interact with the HOX-11 protein (Shen, Chang et al. 1996), suggesting that these may co-operate during splenic development. Brendolan *et al* analysed asplenic mouse models lacking *Pbx-1*, *Hox-11*, *Nkx3.2* or *Pod-1* and found a genetic hierarchy existed, with *Pbx-1* itself playing a common crucial co-regulator role in splenic development (Brendolan, Ferretti et al. 2005). *Hox-11* and *Nkx2.5* were absent in the splenic mesenchyme in their *Pbx-1* -/- embryos. *Hox-11* was completely absent (rather than delayed) in the splenic mesenchyme (*in situ* hybridisations performed from E11-E13.5 embryos) but was present elsewhere in the other organ primordia within these embryos. However, *Pbx-1* expression was not altered in the splenic mesenchyme of *Hox-11* -/- embryos, indicating that the onset and continued expression of *Hox-11* is *Pbx-1* dependent, but not the other way round. Interestingly, although two of the earliest known markers of splenic condensing mesenchyme (*Hox-11* and *Nkx2.5*) were absent, the other splenic mesenchymal

markers *Pod-1* and *Nkx3.2* were maintained in these *Pbx-1* -/- embryos, showing that condensing splenic mesenchymal cells were still present and splenic gene expression is not globally impaired by loss of *Pbx-1*. Interestingly, a TUNEL assay (Gavrieli, Sherman et al. 1992) was performed on this *Pbx-1* -/- mouse splenic mesenchyme and no detectable increase in apoptosis was found, whilst Bromodeoxyuridine (BrdU) *in vivo* labelling showed a marked reduction in the cell proliferation, and thus expansion, of the splenic anlage by E13.5 (Brendolan, Ferretti et al. 2005).

Pbx-1 expression in the splenic mesenchyme is independent of *Pod-1* or *Nkx3.2* in mice embryos, whilst *Nkx3.2* and *Pod-1* are both expressed independently of each other at E12-12.5 (Lu, Chang et al. 2000; Brendolan, Ferretti et al. 2005). Furthermore, *Pbx-1* and *Nkx3.2* both independently regulate *Hox-11* expression, with *Hox-11* expression being dependent on both *Pbx-1* (Brendolan, Ferretti et al. 2005) and *Nkx3.2* (Lettice, Purdie et al. 1999). *Nkx2.5* is not expressed in the splenic mesenchyme of *Pod-1* -/- or *Pbx-1* -/- mice embryos (Brendolan, Ferretti et al. 2005), indicating that its splenic expression is dependent on the expression of these genes. However, *Nkx2.5* is still expressed in the splenic mesenchyme of *Nkx3.2* -/- embryos at E12-12.5 (Brendolan, Ferretti et al. 2005). *Nkx2.5* expression is dependent on both *Pbx-1* and *Pod-1* but not on *Nkx3.2*. Therefore, *Pbx-1* functions upstream of *Hox-11* and *Nkx2.5* and so controls expression of *Pod-1* and *Nkx3.2*, both essential for splenic development (Brendolan, Ferretti et al. 2005).

In addition to genetically regulating downstream expression of key genes controlling splenic expression (*Nkx3.2* and *Pod-1*), *Pbx-1* also genetically interacts with *Hox-11* during splenic development (Brendolan, Ferretti et al. 2005). *Pbx-1* was found to

directly bind to the *Hox-11* promoter, along with *Hox-11* itself, in embryonic mouse splenocytes. The simultaneous binding of *Hox-11* to its own promoter suggests an auto-regulatory role in splenic development.

2.4.3 Wt1

Wt1 (Wilms tumour suppressor gene) is also required for normal splenic development, as *Wt1* -/- mice embryos experience splenic anlage formation around E12 to E13 gestation but the spleen then involutes by E15, before haematopoietic cell invasion (Herzer, Crocoll et al. 1999). *Wt1* is known to be expressed in the condensing splenic mesenchyme (Rackley, Flenniken et al. 1993). *Hox-11* mRNA is first expressed at E10.5 followed by *Wt1* expression at E11.5 (Koehler, Franz et al. 2000). During E11, *Wt1* expression overlaps with *Hox-11* expression in the spleen (Herzer, Crocoll et al. 1999) but its expression is decreased in *Hox-11* -/- embryos, indicating that *Wt1* expression is regulated by *Hox-11* during splenic ontogeny (Koehler, Franz et al. 2000). *Wt1* expression was also down-regulated in the condensing splenic mesenchyme of *Pbx-1* -/- embryos (Brendolan, Ferretti et al. 2005), but was maintained in the outer mesothelial lining (which will give rise to the splenic capsule (Sadler and Langman 1995). Taken together with the *Pbx-1* -/- and *Hox-11* data, a genetic hierarchy has been suggested whereby *Pbx-1* regulates *Hox-11*, which in turn regulates *Wt1* in the condensing splenic mesenchyme (Brendolan, Ferretti et al. 2005). Furthermore, a recent report has shown that absence of *BarH-like homeobox 1* (*Barx-1*) expression (a stomach mesenchymal transcription factor) in mice embryos results in defective splenic positioning and expansion, with reduced splenic *Wt1* expression (Kim, Miletich et al. 2007).

2.4.4 Bapx1 (Nkx3.2)

*Bapx1 (*also known as *Nkx3.2)* is known to be important for normal splenic development. *Bapx1* null embryos die perinatally and, amongst other anomalies such as skeletal dysplasia, are notably asplenic with an absence of splenic precursor cells from an early stage of splenic anlage formation (Lettice, Purdie et al. 1999; Tribioli and Lufkin 1999; Akazawa, Komuro et al. 2000). In the mouse, *Bapx1* is initially expressed in the dorso-lateral mesenchyme flanking the developing pancreas and by E12.5 it is restricted to the splenic anlage, with no expression in the gastrointestinal or pancreatic endoderm (Hecksher-Sorensen, Watson et al. 2004).

2.4.5 Nkx2.5

The homeobox gene *Nkx2.5* serves as a marker for splenic precursor tissue (Patterson, Drysdale et al. 2000). *Nkx2.5* is expressed in high levels in the adult heart and spleen of the mouse (Lints, Parsons et al. 1993) and is known to be expressed in early stages of splenic development in Xenopus (Patterson, Drysdale et al. 2000). Pre-splenic tissue is initially located in symmetric domains on both sides of the embryo in stage 37 Xenopus embryos, but only the left side goes on to form the mature spleen (*Nkx2.5* only detectable on left side by stage 40), possibly through preferential development of spleen precursor cells on that side. In mouse embryos, *Nkx2.5* is expressed at E10.5 in two distinct ventral and dorsal domains around the dorsal pancreatic bud, which overlap *Hox-11* expression (Hecksher-Sorensen, Watson et al. 2004).

2.4.6 Pod-1 (capsulin / epicardin)

Pod-1 is also known as *capsulin* (Hidai, Bardales et al. 1998; Lu, Richardson et al. 1998) or *epicardin* (Robb, Mifsud et al. 1998). This transcription factor is expressed in mesenchymal cells that encapsulate the epithelial primordia of developing viscera, such as kidney, lung and epicardium (Quaggin, Schwartz et al. 1999; Lu, Chang et al. 2000). *Pod-1* is required for splenic development, acting after splenic specification to control expansion of the splenic anlage (Robb, Mifsud et al. 1998; Quaggin, Schwartz et al. 1999; Lu, Chang et al. 2000). *Pod-1* -/- mice fail to form a spleen, with rapid apoptosis of the early splenic primordia in these cases and this region is devoid of cells by E13.5 (Lu, Chang et al. 2000). *Pod-1* expression overlaps with other transcription factors *Nkx3.2*, *Hox-11* and *Nkx2.5* in the splenic mesenchyme (Lu, Richardson et al. 1998; Brendolan, Ferretti et al. 2005).

2.4.7 Nkx2.3

The transcription factor *Nkx2.3* is expressed in the gut mesenchyme and spleen of embryonic and adult mice (Pabst, Zweigerdt et al. 1999). It is essential for normal development and function of the small intestine and spleen. *Nkx2.3* -/- mice have severe morphological alterations in these organs and die in early postnatal life. *Nkx2.3* mutants develop smaller spleens with disorganised white pulp and reduced numbers of lymphatic cells, whilst some are asplenic.

2.5 Close developmental relationship between the spleen, pancreas and stomach

The spleen is known to form from a mesenchymal condensation within the dorsal mesogastrium (the mesenchymal sheet that attaches the stomach to the dorsal body wall), adjacent to the dorsal pancreas and stomach during development (Patterson, Drysdale et al. 2000). The spleen normally displays left-handed asymmetry (Boorman and Shimeld 2002) and the position of this organ has been used in detecting defects of laterality, such as situs invertus (Aylsworth 2001). Interestingly, there is no clear correlation between Left-Right asymmetry and the presence or absence of a spleen, as shown by a host of papers studying laterality defects in mice and humans. These report a range of either normally developed spleens, splenic hypoplasia or asplenia (Yokoyama, Copeland et al. 1993; Roberts, Shutter et al. 1994; Dear, Colledge et al. 1995; Lettice, Purdie et al. 1999; Tribioli and Lufkin 1999; Oh and Li 2002). Also, asplenia may occur in the absence of laterality defects (Rose, Izukawa et al. 1975; Waldman, Rosenthal et al. 1977). Taken together, these findings indicate that mechanisms other than those controlling Left-Right symmetry must be involved in the control of splenic development (Brendolan, Ferretti et al. 2005).

The spleen shares a very close developmental relationship with the pancreas, with the splenic mesenchyme budding off from the pancreatic mesenchyme early in development (Asayesh, Sharpe et al. 2006). This very close developmental relationship makes it an ideal candidate tissue for further investigation in the search for a candidate tissue source for future islet neogenesis. Asayesh *et al* studied this developmental relationship in *Bapx1* -/- mice. In normal development, the splenic mesenchyme initially lies adjacent to the pancreatic endoderm (Hecksher-Sorensen,

Watson et al. 2004). Shortly after E11, the spleen forms as a condensation and buds off from the dorsal pancreas. These tissues are clearly separated by E13.5. However, no splenic condensation and separation occurs in *Bapx1 -/-* mice and the presumptive splenic mesenchyme, which still expresses the splenic mesenchymal markers *Nkx2.5* and *Pod-1* (Brendolan, Ferretti et al. 2005) in these mice, remains as loosely organised cells lateral to the dorsal pancreas. This tissue can then undergo apoptosis (shown with a TUNEL assay by Asayesh *et al*) or it can go on to form smooth muscle (indicated by *c-kit* expression performed by Asayesh *et al*) with the adjacent dorsal pancreatic tissue forming cystic structures which express *sonic hedgehog* (*Shh*), a transcription factor expressed in embryonic gut endoderm and a mediator of gut endoderm and mesoderm development (Ramalho-Santos, Melton et al. 2000) .

This close developmental relationship between the pancreatic mesenchyme and the spleen has also been observed in other murine studies. In mice with defects in late pancreatic development, where the pancreatic mesenchyme is unaffected, the spleen usually survives intact. However, loss of pancreatic mesenchyme is strongly linked with asplenia (Ahlgren, Jonsson et al. 1996; Apelqvist, Ahlgren et al. 1997; Harrison, Thaler et al. 1999; Kim, Miletich et al. 2007).

In mice born without an exocrine pancreas, due to a null allele for the protein PTF1-p48, functional islets are found in the spleen (Krapp, Knofler et al. 1998). The transcription factor *Ptf1* directs expression of genes in the exocrine pancreas. Mice bearing a null mutation die shortly after birth and are found to have a complete absence of exocrine pancreatic tissue, indicating that protein p48 is required for differentiation and / or proliferation of the exocrine cell lineage. Interestingly,

hormone-secreting pancreatic endocrine cell lineages do exist in these mice, in the mesentery normally harbouring the pancreatic organ, until E16. Subsequently, from E16 to E18, these cells are no longer found in this location but are now found in the spleen instead, where they remain functional until neonatal death. It is also interesting to note that naturally occurring giant splenic islets (devoid of exocrine tissue) are found in some reptiles, including rattlesnakes and vipers (Hellerström and Asplund 1966; Volk and Arquilla 1985; Moscona 1990).

The stomach, pancreas and spleen all share a common splanchnopleural mesodermal lineage (Matsushita 1995; Kumar, Jordan et al. 2003), as well as common genetic regulators. Interestingly, Kumar *et al* showed that the mesoderm underlying the presumptive pancreatic domain (splanchnic mesoderm between somites 7-9 at the 10 somite stage) in chick embryos was capable of inducing *Pdx-1* (Gu, Dubauskaite et al. 2002; Gu, Brown et al. 2003) and other pancreatic gene expression in non-pancreatic endoderm. This mesoderm was also able to initiate formation of ectopic islet-like clusters in endoderm that would normally give rise to the stomach (possibly via BMP / activin and retinoic acid mesodermal signals).

Expression of the homeobox gene *Barx-1* is restricted to stomach mesenchyme during gut organogenesis (Tissier-Seta, Mucchielli et al. 1995; Kim, Buchner et al. 2005). However, it is also required for normal formation of the spleen, as studied in mouse embryos (Kim, Miletich et al. 2007). *Barx-1* negative mutants developed normal lower abdominal organs but the spleen was markedly hypoplastic and embedded within the dorsal pancreas, rather than in its usual location (apposed to the greater curvature of the stomach). In addition, the dorsal and ventral pancreatic buds failed to

fuse (possibly due to absence of stomach rotation) in these embryos. The authors (Kim *et al*) report reduced expression of *Wt1* transcription factor in the mesothelium of *Barx-1* -/- mice, but the expression of other transcription factors known to be implicated in spleen development were unaffected (*Pbx-1*, *Tlx-1*, *Nkx2.5*, *Bapx1*, *Pod-1*).

2.6 Clinical observations

There is some evidence from the medical literature indicating that splenic preservation may delay the onset of diabetes in certain illnesses. Firstly, two retrospective reviews (from two different centres) of patients undergoing surgery for chronic pancreatitis found a reduced incidence of diabetes after distal pancreatectomy with splenic preservation compared with distal pancreatectomy with splenectomy (Govil and Imrie 1999; Hutchins, Hart et al. 2002). Secondly, children undergoing splenectomy for severe thalassaemias have an increased incidence of glucose intolerance (Bannerman, Keusch et al. 1967; Lee, Tan et al. 1985). Diabetes Mellitus is a well documented complication of multiple transfused thalassaemias (possibly as a result of gradual iron accumulation), with an estimated overall incidence of 17.5% (Zuppinger, Teuscher et al. 1975). Although no children in the Lee *et al* paper were found to be diabetic, those who had undergone splenectomy had a higher incidence of glucose intolerance. The authors attribute the lack of diabetes detection in their patient cohort to the relatively young group of children studied (age range 13 months-13 years) as it is rare for diabetes to complicate thalassaemia before the age of 10 years, with most reports in patients in their teens and twenties (Lassman, O'Brien et al. 1974; Saudek, Hemm et al. 1977). Taken together, these lines of evidence suggest that the spleen may play a role in delaying or preventing diabetes in pancreatic disease. The mechanism for this

is unclear. The increased incidence of glucose intolerance in children undergoing splenectomy for severe thalassaemias could simply be a direct effect of increased haemosiderin deposition. However, one alternative explanation for these reports of delayed diabetes onset with splenic preservation could be from a possible adult stem cell population residing in the spleen, which may be recruited to form replacement islets / beta-cells.

2.7 Advantages of the spleen as a potential tissue source for future adult stem cell therapeutic strategies

The mature human spleen is an attractive candidate tissue source for future adult stem cell therapeutic strategies to reverse Diabetes Mellitus. Firstly, the spleen has the practical advantages of being a surgically very accessible organ. The spleen can be safely and readily accessed surgically via laparotomy, or via laparoscopy if a minimally-invasive approach was favoured (Rosen, Brody et al. 2002; Khoursheed, Al-Sayegh et al. 2004; Pomp, Gagner et al. 2005; Silecchia, Raparelli et al. 2005; Ruiz-Tovar, Perez-de Oteyza et al. 2007; Makrin, Avital et al. 2008). Splenic tissue could therefore be readily acquired from the diabetic patient themselves, which may overcome the difficulties of donor shortages currently limiting allograft transplantation. It may also alleviate the requirements for immunosuppression when the tissue is autotransplanted back into the host following *in vitro* manipulation. However, it is likely that a concomitant strategy to prevent reactivation of the autoimmune response would also be required. Reduced immunosuppression requirements may also make it available to younger recipients such as children, a group who could gain most benefit from islet transplantation, but who are currently not routinely offered allograft islet transplants owing to the risks associated with

lifelong immunosuppression (Coulombe and Gill 2004; Noguchi, Matsumoto et al. 2006). Alternatively, a splenic source of replacement islets could be used for allotransplantation. It is interesting to note that the spleen is largely discarded at present during multi-organ retrieval, but could be utilised as an abundant allograft tissue source, if spleen-derived islet neogenesis is successfully realised.

Secondly, the spleen is advantageous as it is a "non-essential" organ. If a large tissue mass is required for *in vitro* manipulation, splenectomy could be considered. The patient could survive after a full splenectomy if this amount of tissue was required. However, as mentioned earlier, splenectomy is not without risk of OPSI and so the appropriate immunisations, longterm antibiotics and patient counselling would be necessary. Ideally, only a sample of splenic tissue would be required, and then these cells could be grown and manipulated in an *in vitro* culture system in order to achieve the required islet number prior to auto-transplantation. Smaller splenic samples could be acquired by splenic biopsy, or possibly a partial splenectomy. Splenic biopsy can safely be achieved percutaneously under radiological guidance (Kang, Kalra et al. 2007; Tam, Krishnamurthy et al. 2008). Ultrasound-guided biopsy of the spleen has been reported to be a safe procedure (Civardi, Vallisa et al. 2001; Lieberman, Libson et al. 2003), including its use in children (Muraca, Chait et al. 2001). The spleen also has a natural capacity to regenerate after partial splenectomy, making it a very attractive tissue source to harvest for adult stem cell strategies (Hall, Kurtzberg et al. 2005; Diesen, Zimmerman et al. 2008). It should be noted, however, that despite the potential advantages of using the spleen as a tissue source, any surgical procedure on the spleen does carry a risk of bleeding.

The mature spleen is known to contain an adult stem cell population, capable of extramedullary haematopoiesis at times of stress (Chadburn 2000). When the bone marrow's ability to produce blood cells is exceeded, during disease or at times of stress, the mature spleen can initiate production of any type of blood cell or component via this haematopoietic stem cell population. Although, the exact mechanisms behind this extramedullary haematopoiesis phenomenon are not yet understood, a recent report from mouse model experiments indicates that bone marrow cells can home to the spleen during acute anaemic stress, where a specialised population of erythroid progenitors are generated for erythropoiesis, via a *BMP4* and *Hedgehog* dependent signalling pathway (Perry, Harandi et al. 2008). It is also interesting to note, given this ability, that the embryological spleen is one of the first sites of haematopoiesis during development. At around six weeks of gestation, the developing human spleen produces a full complement of haematopoietic cells. This actually precedes haematopoiesis by the bone marrow, which begins at five months gestation, when splenic production decreases (Chadburn 2000). This known functional adult stem cell population may be a useful potential target for future therapeutic strategies in islet neogenesis (Kodama, Davis et al. 2005).

The mature spleen also appears to be a favoured site for stem cells to reside. Foetal cells from offspring have been found at autopsy in humans (decades after pregnancy) in the mother's spleen (Johnson, Nelson et al. 2001). Foetal cells were found more frequently in the spleen than in other tissues, such as the pancreas or lymph nodes, suggesting that the spleen may be a preferential site for foetal cells to be sequestered. The possible consequences (beneficial or deleterious) of this foetal cell persistence in maternal tissues is not yet fully understood (Johnson and Bianchi 2004). Furthermore,

when pancreatic "stem cells" isolated from the adult human donor pancreas are implanted into immunocompetent mice, these stem cells are subsequently found to naturally reside in the mouse spleen and remain there for at least two months, in preference to other sites such as blood or bone marrow (Abraham, Kodama et al. 2004).

Hox-11 (*Tlx-1*) is known to have widespread expression during embryogenesis (as previously mentioned in Chapter 2, section 2.4.1). However, Kodama *et al* found that a putative mesenchymal stem cell population resides within the spleen of adult mice (Kodama, Davis et al. 2005). This cell population was *Hox-11* +ve, *Pdx-1* -ve and CD45 -ve, the latter indicating that these cells were of non-lymphoid origin. These cells were localised in the subcapsular region of the spleen in mice. As previously mentioned (Chapter 2, section 2.4.1), a reservoir of *Hox-11* (*Tlx-1*) positive stem cells has also recently been found to be uniquely and abundantly expressed throughout adulthood in the human spleen (Dieguez-Acuna, Gygi et al. 2007; Lonyai, Kodama et al. 2008). Over 30 normal human post-mortem spleens were analysed (obtained during harvesting procedures for organ donation) and there was no difference in expression by gender or age. These cells were located throughout the human adult spleen (rather than the predominantly subcapsular region in the mouse). Strikingly, these stem cells displayed autonomous proliferation in tissue culture and were maintained in culture for at least two months, with persistent *Hox-11* (*Tlx-1*) expression. The authors also suggest that the abundant expression of this gene means that these stem cells may not need to be expanded in culture prior to transplantation (Lonyai, Kodama et al. 2008). *Hox-11* expression is already known to play a role in influencing stem cell behaviour, as the artificial over-expression of *Hox-11* in

embryonic stem cells induces the immortalization of embryonic precursors (Keller, Wall et al. 1998) . It is also interesting to note that *Hox-11* plays an important role in nature in tissue regeneration within newts, since persistent up-regulation of a *Hox-11* - like gene in this species contributes to the regeneration of entire limbs and the tail (Simon and Tabin 1993; Beauchemin, Noiseux et al. 1994). Taken together, this evidence suggests that splenic stem cells are very promising candidates for use in future therapeutic transplantation strategies.

2.8 NOD mouse model: role of donor splenocytes in reversal of diabetes?

Non-obese diabetic (NOD) mice are a well-established model for human T1DM. They exhibit spontaneous autoimmunity which causes diabetes through destruction of the Islets of Langerhans. Kodama *et al* have reported permanent reversal of diabetes in NOD mice (with end-stage disease) through injection with adult mice donor splenocytes combined with elimination of the autoimmunity using Freund's complete adjuvant (FCA) (Ryu, Kodama et al. 2001; Kodama, Kuhtreiber et al. 2003). The return of endogenous insulin secretion and persistent restoration of normoglycaemia was accompanied by the reappearance of pancreatic beta-cells. Live or irradiated male adult mouse donor splenocytes were injected into severely diabetic NOD females in these experiments in order to investigate the origin of the new pancreatic islets (Kodama, Kuhtreiber et al. 2003). The mice also received a temporary (40 day) syngeneic islet transplant under the capsule of one kidney to control blood glucose concentration. Interestingly, when this islet implant was removed, 6 of the 9 mice (67%) that received live splenocytes remained normoglycaemic, whilst none of the 8 mice that received irradiated splenocytes remained normoglycaemic (they all became

rapidly and severely hyperglycaemic). In another experiment in this series, the islet transplant was then maintained for 120 days before removal, to allow a longer period for islet regeneration. However, in this experiment, the majority of mice remained normoglycaemic in both the live and irradiated donor splenocyte groups, indicating that both types of splenocytes could effect disease elimination when a longer period of normoglycaemia is imposed. In the successfully treated mice, fluorescence *in situ* hybridisation (FISH) analysis for detection of the Y chromosome of the male donor cells showed abundant nuclei positive for the Y chromosome within the islets, but not the exocrine portion of the pancreas. 29% to 79% of islet cells were of donor origin. No islets solely of host origin were detected and other organs (such as the brain, liver and kidneys) were devoid of the donor Y chromosome, indicating that the incorporation of the donor cells was selective for the diseased pancreas. The absence of markedly enlarged nuclei or tetraploid nucleoli in the regenerated beta-cells suggested that new islet cells did not arise by fusion of donor splenocytes with endogenous islet cells. In addition, the ploidy of the sex chromosomes of regenerated islet cells was normal, also indicating that the regenerated islets were not the result of fusion between male and female cells. A final experiment in this series, using a green fluorescent protein (GFP) marker, examined whether lymphoid (CD45 +ve) or nonlymphoid / mesenchymal (CD45 -ve) donor splenocytes engaged in islet regeneration. This experiment found that donor CD45 -ve mesenchymal precursor cells were reconstituted into functional islets, whilst the donor CD45 +ve splenocytes did not participate directly in islet regeneration (although they were essential for disease reversal). Overall, the experiments in this paper showed that the observed islet regeneration in the pancreas was a combination of regeneration with the donor spleen cells as well as endogenous regeneration of the treated host (possibly via a

donor splenocyte contribution to the reversal of autoimmunity, allowing islet regeneration from endogenous precursor cells). However, it should be noted that although FCA can abolish autoimmunity in mice, the immune reaction produced is currently considered too hazardous for use in humans.

In 2006, the Chong research group reported partial recovery of endogenous islet beta-cell function in streptozotocin-induced diabetic mice, following removal of an 120 day syngeneic islet transplant, through a combination of beta-cell proliferation and hypertrophy (Yin, Tao et al. 2006). Interestingly, they found that splenic preservation, or splenocyte infusion following splenectomy, facilitated the restoration of beta-cell function compared to splenectomised mice. However, they found no evidence of donor splenocytes differentiating into islet beta-cells in their study. Disappointingly, three groups (Chong group included) have now been unable to reproduce Kodama *et al*'s findings in mice (Chong, Shen et al. 2006; Nishio, Gaglia et al. 2006; Suri, Calderon et al. 2006). Chong *et al* used a similar approach to Kodama *et al*, but used splenocytes from a transgenic mouse strain in which the mouse insulin promoter (MIP) drives the expression of GFP, so that GFP is expressed only in their beta-cells and not in their splenocytes. FCA and splenocytes reversed autoimmunity in 9 / 22 (41%) of the diabetic NOD mice. 14 (63.6%) redeveloped diabetes within 40 days, and one within 80 days, due to a recurrence of autoimmunity (shown by a second syngeneic islet transplant given to 6 of these mice, 4 of whom became diabetic again within 1 week). The majority of these mice remained normoglycaemic after removal of the islet transplant. Histological pancreas examination showed 5 / 6 treated mice had very few hyperplastic islets and one had very few small islets that stained very weakly or did not stain for insulin (similar finding to Suri *et al*; 2006). All islets

within the treated NOD mice were examined for expression of GFP, to determine if the origin of the new islets were from the host or derived from donor spleen cells. No beta-cells expressing GFP were detected, nor in the organs outside of the pancreas, and thus this data does not support a conclusion of beta-cell regeneration from spleen derived cells. The authors conclude that the therapeutic protocol tested was sufficient to reverse autoimmune diabetes with restoration of normoglycaemia but via replication of pre-existing beta-cells, rather than from the donor splenocytes themselves.

Nishio *et al* were also unable to reproduce Kodama *et al*'s findings in the NOD mouse model. They found islet recovery and reversal of the murine diabetes but an absence of any infused donor splenocyte contribution to the regenerated beta-cell mass (Nishio, Gaglia et al. 2006). Single Nucleotide Polymorphism (SNP) analysis was used to identify donor versus host cells within the regenerated pancreatic islets, which the authors argue is a more robust test than the FISH analysis used by Kodama *et al.* They found that host beta-cells (or possibly seeding and expansion of islet graft-derived cells), rather than donor splenocytes, contributed to islet regeneration following muting of the autoimmunity by FCA.

Finally, Suri *et al* were also unable to reproduce Kodama *et al*'s findings. In this study, islet regeneration and disease reversal occurred in the treated NOD mice, through control of the autoimmunity rather than via allogeneic splenocyte-derived differentiation of new islet beta-cells (Suri, Calderon et al. 2006). GFP expressing male donor splenocytes were given to female NOD hosts (as per Kodama *et al*'s second protocol) but although all treated mice were protected from diabetes and with

intact islet regeneration, no GFP +ve cells were detected. This showed no evidence for replacement of islets by the allogeneic spleen cells. The authors conclude that residual host beta-cells, or host beta-cell precursors, led to disease reversal and normoglycaemia.

Considerable debate has ensued following the failure of these three separate attempts to replicate Kodama *et al*'s original promising results of donor splenocytes forming new islets (Couzin 2006; Melton 2006). Faustman argues that their cell lineage tracing technique of Y-chromosome FISH to follow transplanted male cells into female recipients was more robust than the GFP cell tagging technique used by Chong *et al*, which can be difficult to detect if the GFP transgene is expressed inefficiently (Faustman, Tran et al. 2006). She also argues that it is also possible for the transgene to be completely silenced in the progeny of GFP-labelled cells (Mezey, Nagy et al. 2003) and that, furthermore, using a non-perfused (frozen) tissue technique could have technical shortcomings since GFP detection requires perfusion of the tissue with fixatives (most commonly formaldehyde-based) to prevent diffusion of the marker out of the cells (Brazelton and Blau 2005). Chong disagrees (in the same publication as Faustman's comment, page 1243b), arguing that GFP is in fact more robust than Y-chromosome FISH analysis in testing a transdifferentiation hypothesis, as it is less prone to artefacts. Chong argues that the Y chromosome FISH analysis can only detect that the cells are male donor cells and not differentiated beta-cells, whereas the MIP-GFP technique would robustly show this, if any such cells were present. However, Faustman's group counter this argument with their use of insulin staining in the same section as the Y chromosome FISH detection to detect islets containing

donor splenocytes. Chong goes on to argue that inefficient expression or inadvertent silencing of GFP expression has not been reported for the MIP-GFP mouse.

Faustman criticises the sensitivity of Nishio *et al*'s SNP method, as the sensitivity was not formally examined and others have reported that this technique cannot be used to detect low levels of chimerism (Reed and Wittwer 2004). Nishio responded (same publication, page 1243c) by arguing that they use a different method than the low sensitivity one cited in the Reed and Wittwer reference and that, more importantly, Nishio *et al* have directly evaluated their assay and found it to have the required sensitivity. They further go on to question the robustness of the Y-chromosome FISH detection method used by Faustman's group, as Faustman reports a false detection of the Y chromosome in untreated mice (Faustman, Tran et al. 2006) but Nishio also additionally criticises inadequate controls (especially an FCA alone control) and poor imaging quality in the Faustman group's paper.

Faustman argues that Suri *et al*'s paper showed the lowest rate of islet function restoration (normoglycaemia), with only 4 out of 22 mice treated successfully. She suggests that Suri *et al* may have had a problem with their mice or the tissue, since the islets of Suri *et al*'s normoglycaemic mice lacked any lymphocytic infiltration in addition to the lack of donor splenocytes, in contrast to all the other reports (Kodama, Kuhtreiber et al. 2003; Chong, Shen et al. 2006; Nishio, Gaglia et al. 2006).

Faustman's group have subsequently further examined their results and found that the age of the NOD mouse at the start of the disease-reversing treatment influenced the degree of splenocyte contribution compared with endogenous regrowth of the islet

(Faustman, Tran et al. 2006). Older mice with more advanced diabetes showed a higher contribution of splenocytes to regenerating islets and so the extent of chimerism in transplanted islets may in part depend on the extent of islet destruction at the time therapy was initiated and the duration of follow-up after therapy. So it is possible that the other groups may have followed up their animals for shorter time periods after therapy and used earlier time points, which may explain their observations of exclusively endogenous islet regeneration.

These seemingly conflicting findings have led to increased scrutiny of the role of the spleen as a potential future tissue source for islet neogenesis. Therefore, the potential role of the spleen to derive replacement islets now warrants further investigation, including an understanding of the molecular mechanisms behind this process and how this phenomenon might be optimised. This was the focus of the research described in this thesis.

2.9 Aims and hypothesis of thesis

The general aims of this thesis were therefore:

1. To investigate whether the developing spleen could differentiate into insulin-producing cells.
2. To investigate the molecular mechanisms behind this iMET process.
3. To investigate how this iMET process could be augmented.

The principal hypothesis addressed in this thesis states that:

Splenic mesenchyme is able to differentiate into an insulin-producing pancreatic endocrine cell fate during development

Seven subsequent hypotheses were generated. These are shown in Table 1 and qualified in their respective chapters.

Table 1 The seven experimental hypotheses generated within this thesis

1. The developing avian spleen is able to differentiate into insulin-producing cells
2. Altering the ratio of splenic mesenchyme to chick pancreatic epithelium in the recombinants will alter the frequency of observed iMET
3. Altering the overall amount of tissue in the recombinants will alter the frequency of observed iMET
4. *Tlx-1* is down-regulated in the splenic mesenchyme of chimaeric recombinant organs
5. *Pdx-1* is up-regulated in the splenic mesenchyme of chimaeric recombinant organs
6. *Isl-1* is up-regulated in the splenic mesenchyme of chimaeric recombinant organs
7. iMET within chimaeric recombinant organs can be augmented by addition of a *Wnt* agonist to the culture medium

(iMET = islet Mesenchyme-to-Epithelial Transition)

The experimental design used to investigate these aims is outlined in the next chapter.

SECTION TWO

Islet Mesenchyme-to-Epithelial Transition

in the developing avian spleen

Chapter Three

Establishing an avian model of pancreatic organogenesis

3.1 Introduction

An avian *in vitro* model of pancreatic organogenesis was used, employing the well-established chick-quail chimaera system to fate-map tissues within recombinants during pancreatic development. In these experiments, quail spleens were recombined with chick pancreatic epithelia. The use of opposing species types for the pancreatic epithelia and splenic mesenchyme mini-organ recombinants allowed robust fate mapping during the analysis stage, since Quail not Chick PeriNuclear (QCPN) is ubiquitously expressed in the nucleoli of all quail tissue.

3.2 Design of *in vitro* model of pancreatic organogenesis

3.2.1 The use of an avian model as a model of human development

The avian model is a well-established robust system for investigating the mechanisms behind human development and disease (Dupin, Ziller et al. 1998; Brown, Hubbard et al. 2003). It is a powerful system for studying development, since it allows experimental embryology to be combined with molecular approaches (Streit and Stern 2001). The human and chick genomes show greater preservation of gene order than between even human and murine genomes (Hillier, Miller et al. 2004). Avian embryonic tissue is readily available and has the ethical advantage of avoiding the need to sacrifice the mother (in accordance with current ethical principles regarding the use of animals in scientific research). The chick genome has 75% homology with the human genome (ICGSC 2004) and the signalling pathways controlling pancreatic differentiation are highly conserved between avian and mammalian species (Kim, Hebrok et al. 1997; Kim, Hebrok et al. 1997; Hebrok 2003; Stafford, Hornbruch et al. 2004; Kume 2005; Kume 2005). Furthermore, there is 68% inter-species homology of

key transcription factors, such as the pancreatic "master gene" *Pdx-1* (Gerrish, Van Velkinburgh et al. 2004). The amino-acid sequences of avian insulin, glucagon and somatostatin are identical to, or closely resemble, those in mammals (Epple and Brinn 1987).

Despite the similarities between avian and human pancreatic development, differences do exist. Firstly, the avian pancreas is compromised of two distinct types of islet: A-islets, in which pancreatic alpha-cells predominate; and B-islets, in which pancreatic beta-cells predominate. B-islets are more numerous and A-islets are larger in size. This results in the avian alpha-cell mass being approximately twice that of the beta-cell mass in post-natal life. The converse is true in mammals, with the beta-cells outnumbering the alpha-cells (McClish and Eglitis 1969; Smith 1974). A less common third islet type exists in the avian pancreas called the "mammalian" islet, because it contains both alpha-cells and beta-cells (Andrew, Rawdon et al. 1994).

Secondly, avian embryos develop two ventral pancreatic buds compared with a single ventral bud in mammalian embryos (Rawdon 1998). However, since our avian *in vitro* model only uses the dorsal pancreatic bud in construction of recombinants, this difference should not affect the validity of our model as a surrogate of human development.

In summary, the avian model is a scientifically valid, ethically sound model with which to test our hypotheses.

3.2.2 Chick-quail chimaeric recombinants as a cell fate-mapping tool

During early development, chick and quail embryos share similar morphology and differentiate into similar structures at similar rates Quail cells transplanted into the differentiating embryonic chick (*in vivo* or *in vitro*) can therefore be considered to develop in register with the chick tissue at that site (Le Douarin 1969). However, quail cells differ from chick cells in that they uniquely and ubiquitously express a quail-specific perinuclear antigen. This allows positive identification of all quail cells in the chick-quail chimaera by immunocytochemistry, using a monoclonal antibody raised against the quail-specific perinuclear antigen (QCPN, Quail not Chick Perinuclear; Selleck and Bronner-Fraser, 1995).

The chick-quail chimaera system provided a highly sensitive and specific cell fate mapping tool to test the hypotheses. It was chosen because of the following advantages:

(a) Ubiquitous expression

This technique's ubiquitous expression of the quail-specific perinuclear antigen ensured that *all* the splenic cells could be identified within the recombinants, to optimise the detection of any iMET in the experiments. Other fate mapping techniques, such as intracellular injection or retroviral transfection of targeted promoter recombination events, often result in smaller numbers of cells being labelled and may result in iMET going undetected.

(b) Uniform intensity of expression

The quail-specific perinuclear antigen is expressed with uniform intensity irrespective of which type of quail tissue is being used or the duration of culture. This has advantages over other techniques such as the lacZ reporter (Percival and Slack 1999), which is weakly expressed in mesenchymal tissue and whose expression intensity may be affected by prolonged duration (and thus may allow iMET to go undetected).

(c) Permanent expression

The quail-specific perinuclear antigen is a permanent cell marker and the signal is not diluted by cell division (as may occur with intracellular injected reporter constructs). It is therefore well suited to modelling the complex and multi-staged process of organogenesis.

(d) No false positive expression

The quail-specific perinuclear antigen is only expressed by quail cells and avoids the potential problem of confounding transfer of the cell label to adjacent unrelated cells, which may occur with fate mapping techniques involving the use of water-soluble dyes to trace cells.

(e) Excellent resolution

This technique provides excellent resolution of the final cell distribution and morphology during analysis.

(f) Established and validated model of pancreatic organogenesis in this laboratory

This chick-quail chimera model has been successfully used previously to fate map cells in pancreatic organogenesis in this laboratory (Jayanthi, Rowan-Hull et al. 2005; Teague, Jayanthi et al. 2005; Teague, Rowan-Hull et al. 2006) and lends itself well to testing our hypotheses.

The chick-quail chimera model does have potential disadvantages:

(a) Requires surgical manipulation

It is important to avoid contamination of quail pancreatic tissue into the chimaeric recombinant when microdissecting the tissue to be used in constructing the chimaeric mini-organs, as this could lead to false positive results. Particular care was taken to address this by using quail spleen controls to test for contamination. In addition, day 4.5 gestation was specifically chosen to harvest the quail spleens as the spleen was distinct from the adjacent quail pancreatic mesenchyme at this stage. Furthermore, meticulous care was taken over the dissection margins when microdissecting the spleen and a safety cuff of quail spleen was typically left behind next to the quail pancreatic mesenchyme before discarding this (see Chapter 4, Table 2).

(b) Possible confounders?

(i) There could theoretically be a possible confounding role of tissue healing after the surgical manipulation, but it is not known if this occurs or indeed if it has any confounding effect at all.

(ii) There could be a possible confounding role of using different species to construct the mini-organs, but this is unknown.

An alternative equally robust technique for fate mapping the spleen during pancreatic organogenesis could have been use of a transgenic mouse model (perhaps using GFP as a cell tracking tool). Time constraints and logistical hurdles precluded their use for this project (but may form part of future research plans).

3.2.3 Dorsal pancreatic bud development as a model of pancreatic development

Dorsal pancreatic bud explants have previously been widely used to model pancreatic development (Le Douarin 1969; Andrew 1975; Apelqvist, Ahlgren et al. 1997; Rawdon 1998; Kramer, Manning et al. 2002; Chiang and Melton 2003; Hald, Hjorth et al. 2003). The dorsal and ventral buds are not identical and the dorsal bud development precedes the ventral, being clearly discernable under the dissecting microscope at an earlier stage. Additionally, differentiation into insulin-positive beta-cells (Kramer, Andrew et al. 1987) and glucagon-positive alpha cells (Hald, Hjorth et al. 2003) precedes that in the ventral buds. Therefore, the preferential use of the dorsal pancreatic bud permits study of pancreatic organogenesis from an earlier stage. HH stage 22-23 (Day 4 gestation) was routinely used in our chimaera recombinants for three reasons; Firstly, the dorsal pancreatic bud is not clearly discernible from adjacent foregut prior to stage 22 and so is difficult to accurately dissect out. Secondly, the differentiating pancreatic bud becomes increasingly lobulated at later developmental stages, which can preclude clean separation of the pancreatic mesenchyme from its epithelium (Gittes and Galante 1993). Finally, this is the stage

previously successfully used in this laboratory to model pancreatic organogenesis (Teague, Jayanthi et al. 2005; Teague, Rowan-Hull et al. 2006). A potential disadvantage of using pancreatic epithelium, without pancreatic mesenchyme, is that signals from the mesenchyme could be lost, which may be important for islet neogenesis.

3.2.4 The avian spleen as a surrogate for pancreatic mesenchyme within the chimaera

The embryonic spleen was used as a surrogate for pancreatic mesenchyme in the chimaeric recombinant organs. The use of a surrogate has been validated in previous studies, for example using stomach mesenchyme (Percival and Slack 1999; Teague, Jayanthi et al. 2005; Teague, Rowan-Hull et al. 2006). As already discussed in Chapter 2 section 4, the spleen shares a very close developmental relationship with the pancreatic mesenchyme, budding off from the pancreatic mesenchyme of the dorsal bud in early development (Yassine, Fedecka-Bruner et al. 1989). This close relationship makes it an attractive surrogate tissue to investigate. The spleen is a mesenchymal structure and therefore lends itself well to use as a mesenchymal surrogate. This avoids the need for a collagenase step to separate mesenchyme from epithelium and affords protection against spurious results from contamination of the mesenchyme by epithelial cells. However, the splenic mesenchyme may have potential disadvantages compared with stomach mesenchyme; Firstly, it is smaller in size than the stomach (which may affect the likelihood of observing any iMET), with the stomach having a thicker mesenchymal investment than the rest of the gut or gut-derivatives (Roberts, Smith et al. 1998). Secondly, the cell contact and proximity of mesenchyme to epithelium in normal pancreatic differentiation during development is

already known to be important (Li, Manna et al. 2004). The stomach lumen maximises cell-cell contact between the stomach mesenchyme and pancreatic epithelium by placing the epithelium explant inside the stomach mesenchymal lumen. Other non-lumenal mesenchyme, such as the splenic mesenchyme, may not benefit from this very close cell-cell contact.

Although there is a paucity of developing avian embryonic spleen descriptions in the literature, a chick atlas and previous ontological study of avian embryonic spleens by Yassine (1989) allowed identification of the spleen during establishment of the model. The splenic tissue being harvested was also confirmed to be splenic by *in situ* hybridisation experiments using *Tlx-1* (see Chapter 4, Tables 2 and 3).

3.2.5 Three-dimensional culture system

The three-dimensional culture system is acknowledged to be superior to two-dimensional systems for studying complex developmental systems (such as the pancreas), as it more accurately models the three-dimensional niche of the developing organ (Affolter, Bellusci et al. 2003). In fact, the microgravity conditions afforded by roller culture is known to enhance pancreatic differentiation and growth during *in vitro* culture (Rose, Brown et al. 1999).

3.3 Validation of *in vitro* model of pancreatic organogenesis

The three-dimensional roller culture apparatus in this laboratory was already known to successfully support pancreatic organogenesis from previous experiments investigating stomach mesenchyme. It was therefore considered to be the optimal culture system with which to now investigate the role of iMET using splenic mesenchyme. Non-recombinant splenic mesenchyme atrophied and dissociated when cultured alone without epithelia. This is a similar finding to that observed in this laboratory previously using stomach mesenchyme alone (unpublished data). Therefore, spleen controls were either fixed after one day culture (after overnight agarose culture step), or fixed straight away after harvesting and microdissecting the embryos at day zero of culture. Importantly, microdissected control spleens were always negative for insulin, *Pdx-1* and *Isl-1* (see Chapter 4, Table 3). This demonstrated clean dissection margins (with no quail pancreas contamination of the quail spleens).

3.4 Conclusion

The *in vitro* model provides a powerful tool for investigating the role of iMET in the developing avian spleen and is a model well-supported by the literature.

Chapter Four

Investigation of Islet Mesenchyme-to-Epithelial Transition in the developing avian spleen during pancreatic organogenesis

4.1 Introduction

The human spleen may be an ideal source of adult stem cells to use for islet transplantation to treat Diabetes Mellitus, as discussed in Chapter 2. However, there has been a great deal of scrutiny over the role of the spleen in achieving this, following conflicting results in the literature of islet neogenesis from donor splenocytes in NOD mice (Kodama, Kuhtreiber et al. 2003; Chong, Shen et al. 2006; Melton 2006; Nishio, Gaglia et al. 2006; Suri, Calderon et al. 2006). Therefore, the potential role of the spleen to derive replacement islets now warrants further investigation. Following controversy in the murine model, it is useful to now investigate splenic iMET in the avian model instead.

I wished to test three hypotheses in the series of experiments described in this chapter:

1. *The developing avian spleen is able to differentiate into insulin-producing cells*
2. *Altering the ratio of mesenchyme to epithelium in the recombinants will alter the frequency of iMET observed*
3. *Altering the overall amount of tissue in the recombinants will alter the frequency of iMET observed*

4.2 Methods

4.2.1 Tissue procurement

Fertile chick (*Gallus domesticus*, hybrid Joice/Hill; Winter Egg Farm, Royston, Herts, UK) and quail (*Coturnix coturnix japonica*; Fayre Game, Lytham, UK) eggs were incubated at 37°C in a humid environment for the required gestation. Developmental

stage of the embryos was determined according to Hamburger and Hamilton staging (Hamburger and Hamilton 1951); see Appendix IV. The embryos were removed from the eggs and placed in chilled (4°C) Leibovitz's L-15 medium (Invitrogen, Paisley, UK). All embryonic manipulation was performed under sterile conditions and in accordance with United Kingdom Home Office regulations under the Animals (Scientific Procedures) Act 1986.

In each experiment, developing embryonic quail spleens E4.5 (Hamburger-Hamilton (HH) stage 25-29) and chick dorsal pancreatic buds E4 (HH22-24) respectively were procured, unless otherwise stated (see Figure 6). Previous experiments in this laboratory, using avian embryonic stomach mesenchyme, have found a window of competency between stage HH22 and HH28 (inclusive) for the mesenchyme to undergo iMET, with a diminishing of this competency with increased gestation (Teague, Rowan-Hull et al. 2006). However, in this thesis, HH25-29 was chosen to microdissect the spleen, as it is morphologically distinct at this stage from the adjacent developing dorsal pancreatic bud. HH22-24 was chosen for the chick dorsal pancreatic buds, as this is the established stage of competency for islet Mesenchyme-to-Epithelial Transition (iMET) previously used in this laboratory, as discussed in Chapter 3, section 3.2.3 (Teague, Rowan-Hull et al. 2006). The microdissections were performed in Leibovitz's L-15 medium using a Leica MZ-16 dissecting microscope and electronically sharpened tungsten needles.

Figure 6 Microdissection of embryos

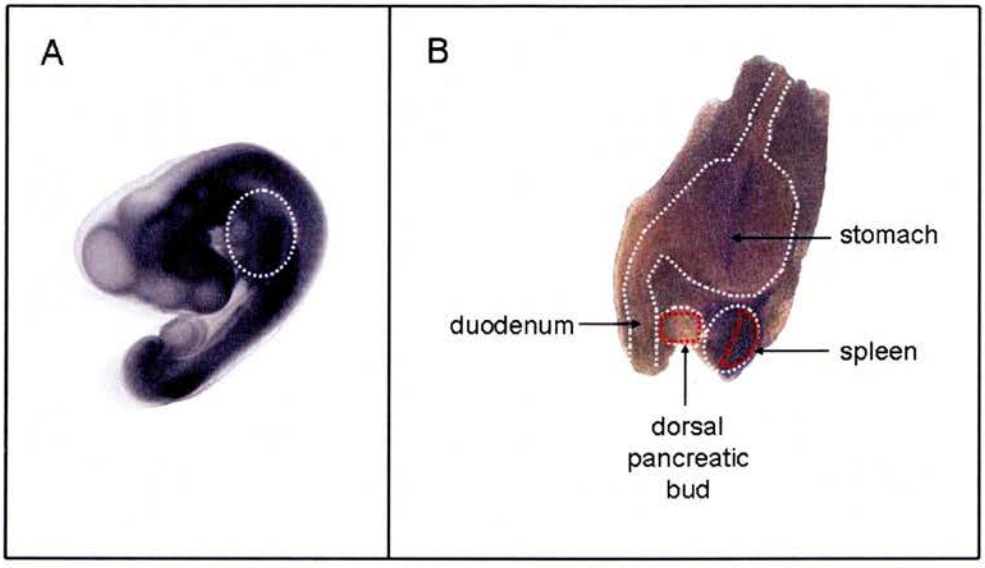


Fig 6 Anatomical boundaries and dissection margins of embryos. Organ blocks were dissected from HH22-24 chick embryos (A; lateral view) and HH 25-29 quail embryos (B; ventral view). White lines indicate anatomical boundaries. Red lines indicate the dorsal pancreatic bud and spleen dissection margins. The dorsal pancreatic bud was taken from chick embryos and the spleen from quail embryos. Particular care was taken over the quail spleen dissection margins, leaving behind a cuff of spleen closest to the pancreas, in order to avoid any quail pancreas contamination.

4.2.2 Separation of embryonic epithelium and mesenchyme

The embryonic epithelium of each pancreatic bud was microdissected from its surrounding mesenchymal cortex using chemical digestion with collagenase, as previously described in the literature (Lear, Jayanthi et al. 2004; Teague, Jayanthi et al. 2005). The pancreatic buds were treated with freshly prepared 0.1% Type A Collagenase (Sigma, Dorset, UK) in Leibovitz's L-15 medium at room temperature

for 8.5 minutes. Following this, the pancreatic buds were transferred into chilled fresh Leibovitz's L-15 medium (without collagenase) and incubated for 1 hour to allow the epithelium and mesenchyme to separate. The epithelium was then microdissected off the mesenchyme. The E4.5 embryonic spleen rudiments required no collagenase digestion and separation, as this is a mesenchymal structure (without an epithelial component to remove).

4.2.3 Chick-quail chimaera recombinant mini-organs

To trace cell lineage during pancreatogenesis, the well-established and highly sensitive chick-quail chimaera model was used. A quail-specific perinuclear antigen is uniquely and ubiquitously expressed by all quail cells, which allows immunocytochemical distinction between cells of quail and chick origin (Le Douarin 1969).

Chimaeric chick / quail embryonic recombinants were initially constructed by recombining one embryonic quail spleen with one chick pancreatic epithelium. Pancreatic epithelium is known to be an important constituent within the recombinant in order for iMET to occur, from previous experiments in this laboratory studying pancreatic mesenchyme (Jayanthi, Rowan-Hull et al. 2005). The amount and ratio of splenic mesenchyme to chick pancreatic epithelium was subsequently altered (where indicated in the sub-sections) to investigate the effect of this on any observed iMET.

With regard to species choice within the recombinant chimaeras, quail tissue was used for the splenic mesenchyme and chick tissue for the pancreatic epithelium because

previous work in this laboratory recombining stomach mesenchyme with pancreatic epithelium, or pancreatic mesenchyme with pancreatic epithelium, found an increased overall incidence of iMET when quail species were used as the mesenchymal tissue rather than chick species (Lear, Jayanthi et al. 2004). In that study, a total of 110 recombinants were constructed, recombining pancreatic mesenchyme with pancreatic epithelium in 70 recombinants and recombining stomach mesenchyme with pancreatic epithelium in 40 recombinants. Within each of these groups, half the recombinants used quail mesenchyme and half used chick mesenchyme, in order to compare any difference between the two species. Overall, 55% of islets were of a mesenchymal origin when quail mesenchyme was used, whilst only 11% of islets were of a mesenchymal origin when chick mesenchyme was used.

4.2.4 In vitro tissue culture

Each recombinant organ was first incubated overnight *in vitro* in a "two-dimensional" ("2-D") culture environment using 1% agarose in culture medium, covered with 2mls culture medium in a humid environment containing 5% carbon dioxide (CO_2) and 95% air at 37°C using a Galaxy R CO_2 incubator (RS Biotech, Ayr, UK). Unless stated otherwise, culture medium was always RPMI 1640 medium containing 10% foetal calf serum, 1% l-glutamine, and 0.3% penicillin / streptomycin (media and all additives from PAA Laboratories, Linz, Austria). This overnight 2-D culture step allowed the constituent chick and quail tissues to join together to form a cohesive chimaeric recombinant mini-organ.

Further *in vitro* culture of the recombinants were then performed in a "three-dimensional" ("3-D") roller culture apparatus (see figure 7) to complete a total culture period of 7 days (modified after New and Cockroft, 1979). Roller culture permits true 3-D growth in microgravity conditions. Both 3-D growth (Affolter, Bellusci et al. 2003) and microgravity conditions (Spooner, Hardman et al. 1994; Rose, Crisera et al. 1999) have advantages over 2-D culture systems for studying complex tissue development (such as the complex branching morphogenesis occurring during pancreatic development). Each recombinant was transferred into its own individual cylindrical glass bottle within the roller culture apparatus (BTC Engineering, Cambs, UK). Each cylinder contained 4mls culture medium, which was exchanged for fresh media every other day. Media were confirmed negative for exogenous insulin (<5.6 picomol/L), glucagon (<5.0 picomol/L) and amylase (<2 IU/L; Department of Clinical and Laboratory Sciences, University of Oxford, UK) prior to use. The roller culture apparatus rotated at 30rpm with continuous gas flow (atmospheric conditions as above).

Figure 7 Roller culture apparatus

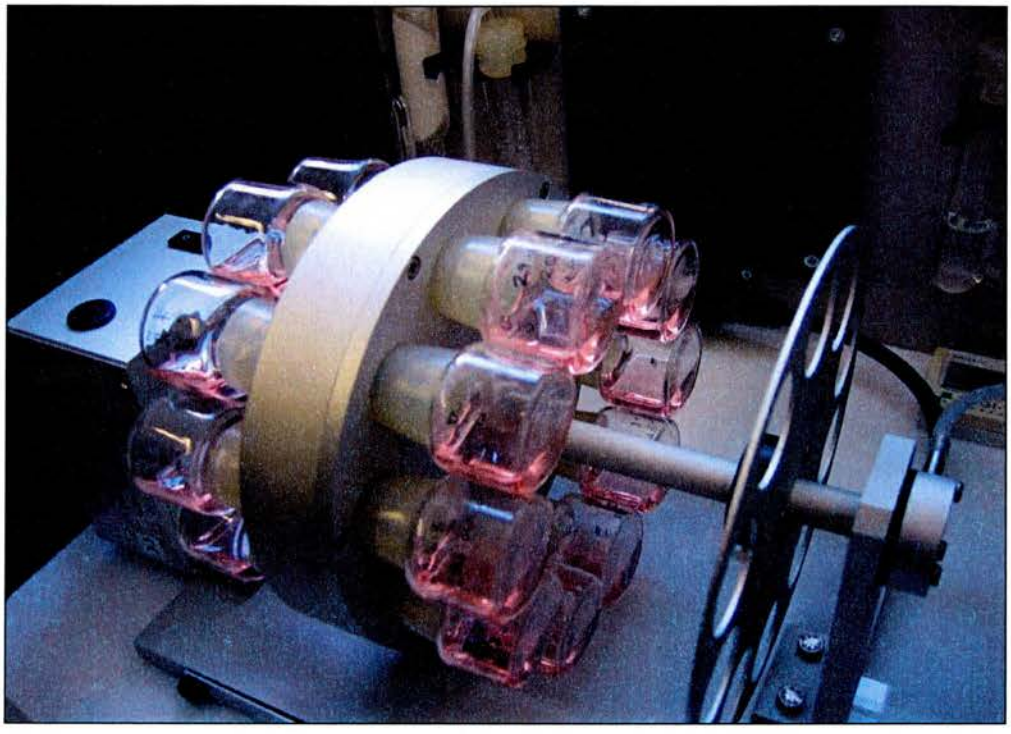


Fig 7 Roller culture apparatus (BTC Engineering). Cylindrical glass containers containing culture medium are shown mounted on a rotation frame (30rpm). The rotating frame is fenestrated to allow continuous gas flow.

4.2.5 Immunocytochemistry

Following *in vitro* culture, tissue was fixed for 2 hours using freshly-thawed phosphate-buffered 4% paraformaldehyde (PFA) with addition of ethylene glycol tetra-acetic acid (EGTA) 2mM, before washing in PBS. Tissue intended for immunocytochemistry on sections was then incubated overnight with 30% sucrose in PBS before frozen-embedding in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Finetek, Thatcham, UK). Frozen tissue was sectioned at 7–10 μm using a Bright cryostat (specimen temperature -18 °C ; chamber temperature -25 °C) and placed on polysine-coated microscope slides (vWR International, Leics, UK). The immunocytochemistry protocol was performed over three (or four) days:

Day One

The frozen sections (on polysine-coated slides) were thawed at room temperature and rehydrated by incubation in PBS, before rinsing with PBT (Calcium Magnesium Free-Phosphate Buffered Saline (CMF-PBS) containing 0.1% Triton-X). Slides were then incubated in blocking buffer for 1 hour at room temperature. Blocking buffer consisted of CMF-PBS containing 5% heat-inactivated goat serum, 0.5% Triton-X, 0.1% Tween-20 and 0.2% Bovine Serum Albumin. Slides were incubated overnight in pre-absorbed primary antibody diluted in blocking buffer at 4 °C.

Day Two

The next day, the primary antibody was washed off with PBT washes and then incubated in blocking buffer for 1 hour before further incubation overnight in fluorescent pre-absorbed secondary antibody in blocking buffer at 4°C.

Day Three

Following overnight secondary antibody incubation, tissues were thoroughly washed (for up to 24 hours) in PBT before mounting in the nuclear stain 4,6-diamidine-2-phenylindole dihydrochloride (DAPI; Roche, West Sussex, UK).

Primary antibodies: (1) mouse anti-quail antibody (QCPN 1:5 ; Developmental Studies Hybridoma Bank [DSHB], IA, USA); (2) guinea-pig anti-insulin (1:2000 ; Sigma, Dorset, UK); (3) rabbit polyclonal anti-ISL antibody (1:200; Abcam, UK). The mouse anti-quail antibody, developed by BM and JA Carlson was obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA.

Secondary antibodies: (1) Alexa Fluor goat anti-mouse 594 (red) or 350 (blue; 1:1000 or 1:2000 ; Molecular Probes, Invitrogen, Paisley, UK); (2) Alexa Fluor goat anti-guinea-pig 488 (green; 1:1000 or 1:2000 ; Molecular Probes, Invitrogen, Paisley, UK); (3) Alexa Fluor goat anti-rabbit 594 (red; 1:1000 or 1:2000 ; Molecular Probes, Invitrogen, Paisley, UK).

4.2.6 Fluorescence tissue microscopy

High resolution images (1024x1024 pixels) were captured using a Hammamatsu Orca digital camera on a Zeiss Axioskop fluorescent microscope.

4.2.7 Analysis of insulin-producing cells

Slides were analysed using fluorescence microscopy for antigen expression and interpreted together with morphological appearance. Sections were cut at 7-10μm thickness (approximately one cell thickness), unless stated otherwise. This was performed in order to attribute antigen expression to the correct cell and also to ensure that only those cells whose morphology and expression were in the sharpest focus in any one focal plane were scored with respect to each antigen.

Embryonic origins of splenic insulin-producing cells were determined according to co-expression of cytoplasmic insulin surrounding a QCPN nucleolus (within a 4,6 – diaminobenzidine tetrahydrochloride (DAPI) nucleus). The QCPN stains the nucleolus of all quail cells, and since all quail tissue was splenic in origin in these experiments, we can identify insulin-producing cells of splenic origin in the recombinants.

An initial analysis was performed, counting the number of recombinants containing insulin-producing cells of splenic origin for each recombinant type. A further detailed analysis was then performed of the number and origin of discrete insulin-producing cell clusters within the recombinants. The embryonic tissue origins of insulin-producing cells within all recombinant mini-organs and controls were classified as epithelial, mesenchymal, or mixed origin. Insulin-producing cell (IPC) clusters of mixed origin imply some epithelial and some mesenchymal cellular contribution to the cluster.

A statistical analysis of the IPC clusters in each group was performed. The data was of a binomial distribution. 95% confidence intervals of obtaining the true proportion of IPC clusters with a mesenchymal contribution in our samples (including purely mesenchymal origin and mixed origin) were calculated for each group. Following this, the proportion of IPC clusters with a mesenchymal contribution in each group of recombinants were compared (two-tailed tests of significance) to test the null hypothesis that there is no significant difference between the proportion in each group. Statistical significance was set at the 5% level.

4.3 Controls

An *in vitro* control for islet cell differentiation was provided by the differentiating pancreatic epithelium within the recombinants. It was also essential to ensure and confirm no contamination of the microdissected quail spleens with quail pancreas, in order to prevent false positive results. This was achieved firstly by taking great care during the microdissection steps to ensure clean separation (as previously discussed in Chapter 3, section 3.2.2). In addition, a cuff of splenic tissue was left behind, attached to the pancreatic mesenchyme, to ensure no pancreatic tissue contaminated the splenic explants. Control for contamination was performed by removing representative microdissected chick pancreatic epithelia and quail spleens at day zero. These underwent overnight culture under the same conditions as the recombinants before undergoing *in situ* hybridisation with the *Tlx-1* probe (as a splenic marker; see Figure 8) and antibody immunocytochemistry for insulin (as a pancreatic marker). The *in situ* hybridisation protocol is described in Chapter 5, section 5.2.7. Microdissected quail spleens underwent simultaneous sectioning and immunocytochemistry steps with the recombinants. In addition, the microdissected unused quail pancreas was also

analysed before discarding this, to give an extra level of confidence over the safety of the dissection margins. The chick pancreatic epithelium and the discarded quail pancreatic tissue underwent the same procedure. Table 2 shows that the dissected control quail spleens were positive for *Tlx-1* and negative for insulin, confirming clean separation, whereas the chick pancreatic epithelium was negative for *Tlx-1* and positive for insulin. Furthermore, the discarded quail pancreas contained a small cuff of *Tlx-1* positive splenic tissue on its distal aspect, giving additional confidence over the safety of the dissection margins. Table 3 summarises all the splenic control data in this thesis, using insulin, *Tlx-1*, *Pdx-1* and *Isl-1*. This demonstrates no quail pancreatic contamination.

Figure 8 Example of an embryonic quail spleen following *Tlx-1 in situ* hybridisation

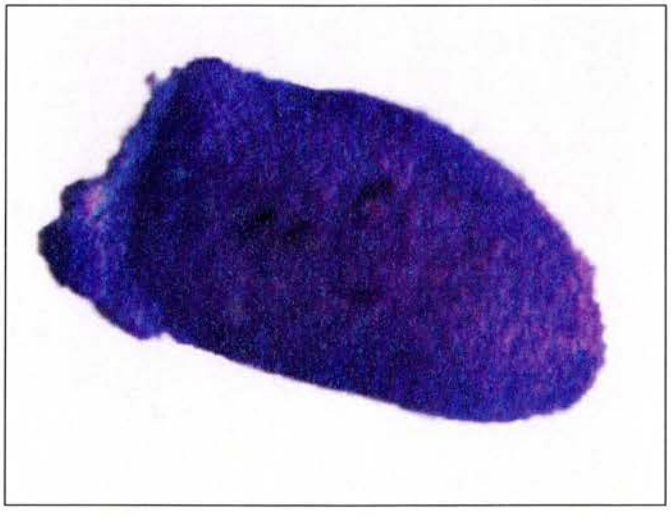


Fig 8 Photograph of a microdissected embryonic quail spleen following wholemount *Tlx-1 in situ* hybridisation. The blue indigo colour indicates a positive hybridisation with the *Tlx-1* probe, confirming splenic tissue. Original magnification x4.

Table 2 iMET control data

	Splenic marker (*Tlx-1 in situ* hybridisation)	**Pancreatic marker** (insulin antibody)
Chick Pancreatic Epithelium	− (n=8)	+ (n=8)
Quail Spleen	+ (n=6)	− (n=39)
Quail Pancreas	+ (n=4)	+ (n=4)

Table 2 *Tlx-1 in situ* hybridisation and insulin antibody immunocytochemistry of controls demonstrating no contamination of dissection margins, confirming clean separation. The quail spleens were all negative for insulin, confirming no pancreatic contamination. A small cuff of quail spleen was also left on the residual quail pancreas before discarding this (*Tlx-1* positive quail pancreas), giving additional confidence over the safety of the dissection margins.

Table 3 Total summary of the quail spleen control data in this thesis

Marker	**Number of + ve quail spleens**	**Number of - ve quail spleens**
Insulin antibody	0	39
Pdx-1 in situ	0	11
Tlx-1 in situ	17	0
***Isl-1* antibody**	0	6

Table 3 The number of microdissected quail spleen controls is shown for each experiment, confirming clean separation. No insulin, *Pdx-1* or *Isl-1* was detected in any control quail spleens, demonstrating no quail pancreas contamination.

4.4 Results

The results are summarised in Table 4. Examples of the insulin-producing cell clusters of splenic origin seen under fluorescent microscopy are shown in Figures 9 and 10.

4.4.1 One quail spleen to one chick pancreatic epithelium recombinants

Recombining one quail spleen with one chick pancreatic epithelium yielded insulin-producing cells (IPCs) of splenic origin in 5 of 12 recombinants (42%). A subsequent detailed analysis revealed that the total number of IPC clusters in this group was 73, giving an average value of 6 per recombinant. The majority of IPC clusters were of an epithelial origin (63%), as would be expected from the pancreatic epithelia within the recombinants. However, 14 (19%) were of a purely splenic origin and 13 (18%) were of a mixed origin, giving 37% overall with a splenic contribution in this group (95% confidence interval = 0.37 ± 0.12). This was significantly more than the two quail spleens to one pancreatic epithelia recombinant group (37% vs 22%; $p = 0.036$) and also significantly more than in the two quail spleens to two pancreatic epithelia recombinant group (37% vs 13%; $p = 0.0014$).

4.4.2 Two quail spleens to one chick pancreatic epithelium recombinants

Recombining two quail spleens with one chick pancreatic epithelium yielded IPCs of splenic origin in 3 of 9 recombinants (33%), a similar proportion to that found in the one quail spleen to one chick pancreatic epithelium recombinant group (see Figure 11). The total number of IPC clusters in this group was 94, giving an average value of 10 per recombinant (see Figure 12). Again, the majority of IPC clusters were of an

epithelial origin (78%), as expected. 18 (19%) were of a purely splenic origin and 3 (3%) were of a mixed origin, giving 22% overall with a splenic contribution in this group (95% confidence interval = 0.223 ± 0.09). There was no significant difference between this value and the 13% found in the two quail spleen to two chick pancreatic epithelia group ($p = 0.139$).

4.4.3 Two quail spleens to two chick pancreatic epithelia recombinants

Recombining two quail spleens with two chick pancreatic epithelia yielded IPCs of splenic origin in 4 of 5 recombinants (80%). This is approximately double the percentage found in the one quail spleen to one chick pancreatic epithelium recombinant group (42%). The total number of IPC clusters in this group was 62, giving an average value of 12 per recombinant. The majority of IPC clusters were again of an epithelial origin (87%). 3 (5%) were of a purely splenic origin and 5 (8%) were of a mixed origin, giving 13% overall with a splenic contribution in this group (95% confidence interval = 0.129 ± 0.092).

Figure 9 Insulin-producing cell cluster of splenic origin within an islet morphology (Example 1)

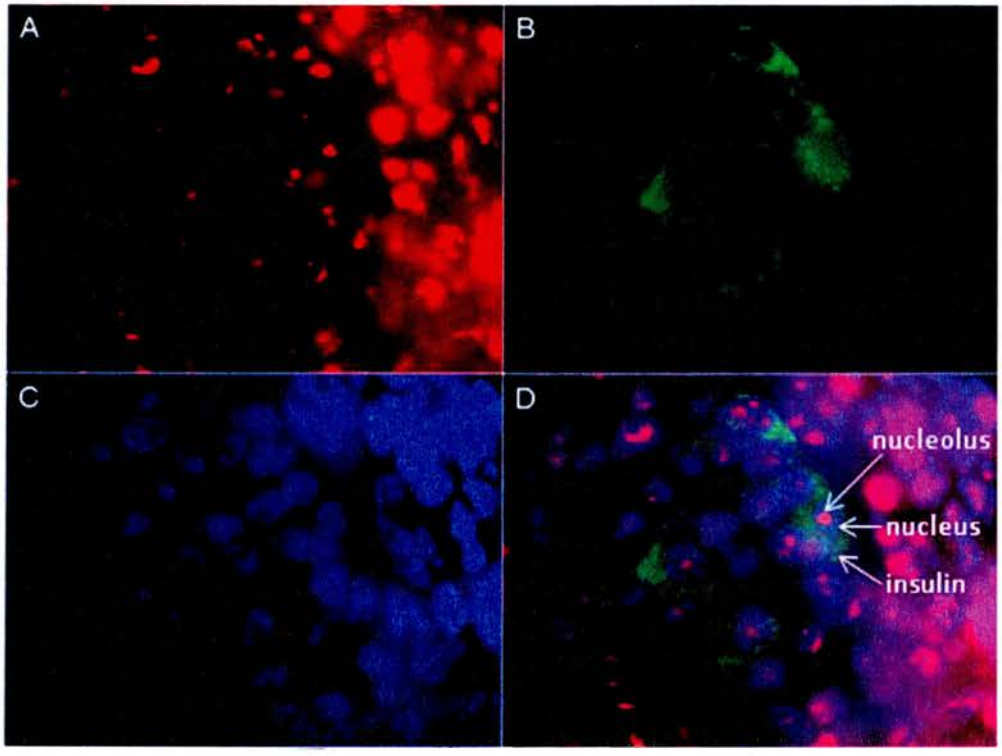


Fig 9 Fluorescent immunocytochemistry of a splenic recombinant showing an example of an IPC cluster of splenic origin within an islet morphology. A: Splenic quail nucleolar QCPN in red, B: Cytoplasmic insulin in green, C: Nuclear DAPI in blue, D: Combined image showing all three channels. This insulin-producing cluster of cells (B) is of splenic origin, as their nucleoli are positive for QCPN (A). Original magnification x63; section thickness 7-10μm.

Figure 10 Insulin-producing cell cluster of splenic origin within an islet morphology (Example 2)

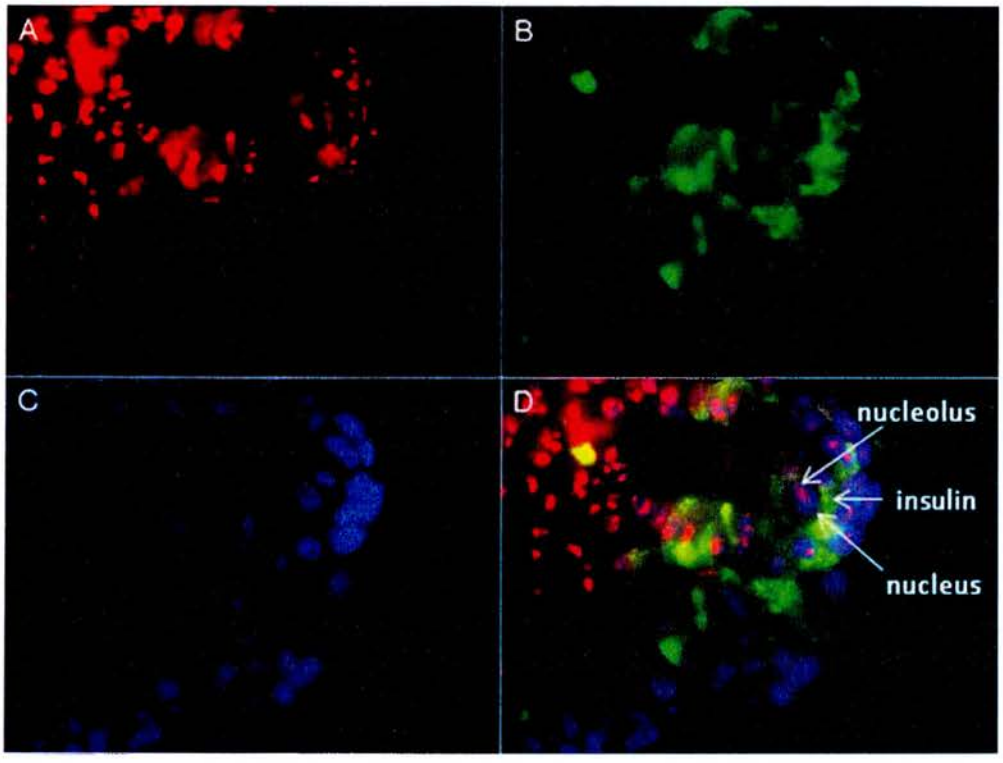


Fig 10 Fluorescent immunocytochemistry of a splenic recombinant showing an example of IPCs of splenic origin within an islet morphology. A: Splenic quail nucleolar QCPN in red, B: Cytoplasmic insulin in green, C: Nuclear DAPI in blue, D: Combined image showing all three channels. This insulin-producing cluster of cells (B) is of splenic origin, as their nucleoli are positive for QCPN (A). Original magnification x63; section thickness 7-10μm.

Figure 11 Bar-chart showing the percentage of recombinants containing IPCs of splenic origin for each type of recombinant ratio

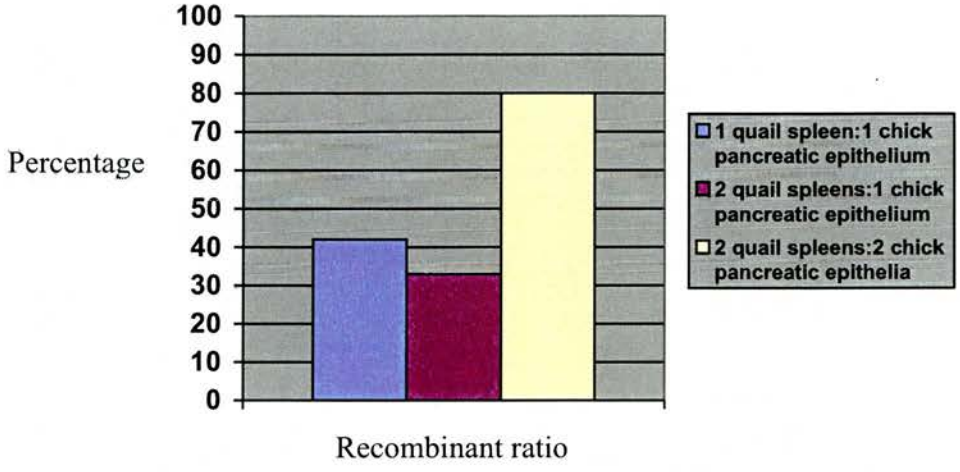


Figure 12 Bar-chart showing the % IPC clusters of each origin for each type of recombinant ratio

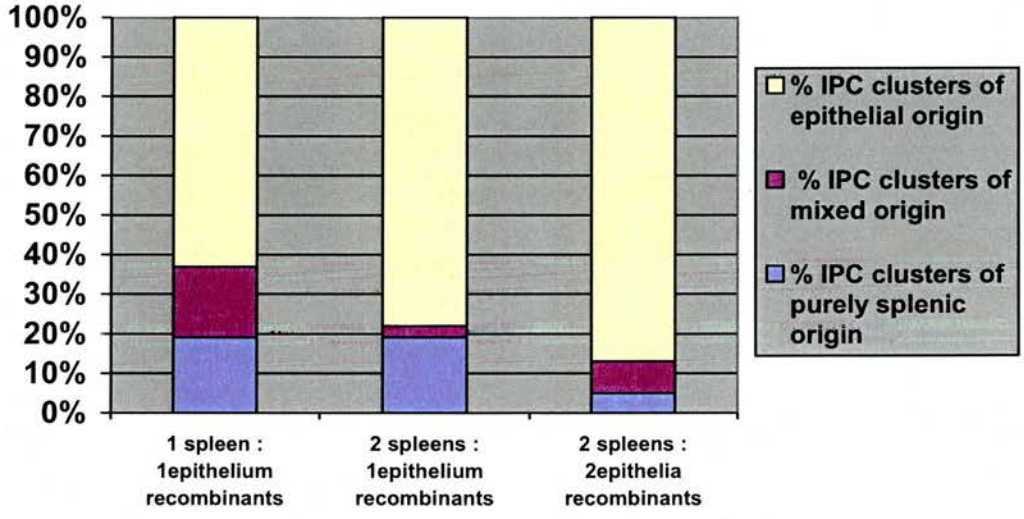


Table 4 Summary table of insulin-producing cell data for each recombinant type

	1 spleen: 1 epithelium recombinants	**2spleens: 1 epithelium recombinants**	**2 spleens: 2 epithelia recombinants**
Number of recombinants	12	9	5
Number of recombinants containing IPCs with a splenic origin (purely splenic or mixed)	5 (42%)	3 (33%)	4 (80%)
Total number of IPC clusters	73	94	62
Average number of IPC clusters/ recombinant	6	10	12
IPC clusters of purely splenic origin	14 (19%)	18 (19%)	3 (5%)
IPC clusters of mixed origin	13 (18%)	3 (3%)	5 (8%)
IPC clusters of epithelial origin	46 (63%)	73 (78%)	54 (87%)
% of IPC clusters with a splenic origin (purely splenic or mixed origin)	37%	22%	13%

4.4 Discussion

Overall, 12 of the total 26 recombinants (46%) contained IPCs of splenic origin, showing that *the developing avian spleen is able to differentiate into insulin producing cells.* Furthermore, it is reasonable to suspect that these discrete IPC clusters represent avian B-islets. These data support previous findings in this laboratory that foregut mesenchymal tissue is able to differentiate into islet tissue (Lear, Jayanthi et al. 2004). The ratio of splenic mesenchyme to chick pancreatic epithelia does not appear to influence the percentage of recombinants in which this is observed (42% vs 33%), suggesting that signals from the differentiating pancreatic epithelium may be a key factor in this iMET process, as this remained the same in these two groups. However, doubling the amount of each tissue proportionately increases the percentage of recombinants in which this is observed (42% vs 80%). A decision was made to construct all subsequent recombinants in this thesis using two quail spleens to two chick pancreatic epithelia based on this preliminary data, showing that the frequency of recombinants experiencing iMET increased proportionately. However, a detailed subsequent analysis of the numbers of discrete IPC clusters within each recombinant group later revealed that the percentage of IPC clusters with a splenic origin contribution significantly decreased when either the ratio of mesenchyme to epithelium, or the amount of tissue in each recombinant, was increased (37% vs 22% vs 13%; see Figure 12). Therefore, although splenic iMET is observed in a proportionately higher percentage of recombinants when the amount of each tissue is increased, the actual percentage of IPC clusters in which iMET is observed significantly decreases. Therefore, constructing recombinants with two spleen and two epithelia may not be the optimal combination for future experiments.

These data show that *altering the ratio of mesenchyme to epithelium in the recombinants alters the frequency of iMET observed,* and *altering the overall amount of tissue in the recombinants alters the frequency of iMET observed.*

These findings could be attributed to altered signalling between the two tissue types within these recombinants. When the amount of each tissue is increased per recombinant, it is reasonable to postulate that with increased tissue mass there are more signals from the increased amount of pancreatic epithelium, and more splenic mesenchyme upon which those signals can act. This might explain the increased chance of iMET occurring in those recombinants (hence iMET observed in 42% vs 80% of recombinants). However, the actual decrease in iMET observed when IPC clusters are counted suggests that this signalling process is more complex. It is possible that the pancreatic epithelial signals can only reach a smaller amount of mesenchyme in the larger sized recombinants, possibly through a reduced interface between the two tissue types. Alternatively, the pancreatic epithelial signals may have limited action on tissue that is not in direct contact with it and it is possible that the contact between the mesenchyme and epithelium may be reduced in the larger recombinants. It seems unlikely that the decrease in iMET is due to altered culture conditions (lack of glucose or nutrient availability in the culture media, for example) when the recombinant size increased, since the average number of IPC clusters (irrespective of origin) per recombinant proportionately increased (an average of 6, 10, 12 IPC clusters per recombinant respectively). This proportionate increase would be expected, as the amount of tissue per recombinant in each group is increasing, although the iMET contribution itself decreases.

An alternative explanation for these findings could be that some signalling is coming from residual chick pancreatic mesenchymal tissue within the recombinant, rather than coming exclusively from the pancreatic epithelia. Although great care was taken during recombinant construction to cleanly separate chick pancreatic epithelia from the chick pancreatic mesenchyme, some pancreatic mesenchymal cells may have remained. Additional control studies would be required to demonstrate clean separation of these tissues in order to investigate this. A further alternative explanation to consider for these findings could be that splenic mesenchyme and pancreatic epithelial cells within the recombinants undergo spontaneous fusion. However, this is a very rare occurrence both in the chick-quail chimaera system (Grzeschik 1973) and in mesenchymal beta-cell differentiation (Ianus, Holz et al. 2003), and would not account for the frequency of splenic iMET observed (discussed further in Chapter 10).

Interestingly, the stomach, pancreas and splenic mesenchyme share a common splanchnopleural mesodermal lineage (Matsushita 1995; Kumar, Jordan et al. 2003) as well as common genetic regulators. As a result, the meso-epithelial relationship in patterning these organs has been an area of intensive investigation for potential therapeutic strategies. Interestingly, *Hox-11* / *Tlx-1* knockout mice are born without a spleen and have a larger stomach and possibly pancreas, suggesting that the splenic mesodermal cells contribute to these other organs when this gene is absent (Roberts, Sonder et al. 1995). Recent experiments examining stomach mesenchyme have shown that splenic rudiments become embedded within the pancreas if *Barx-1* (a stomach mesenchymal transcription factor) is knocked out (Kim, Miletich et al. 2007).

Furthermore, the pancreatic and splenic mesenchymal marker *Bapx1* is required for correct separation of these two organs (Asayesh, Sharpe et al. 2006).

The process of iMET seen in these experiments could be explained by a possible stem cell population in the mesenchyme common to these tissues, which under particular microenvironmental conditions is able to reprogram itself and pursue a fate other than the tissue-specific one. It has also been suggested that non-haematopoietic stem cells reside in the spleen (Chadburn 2000). Indeed, spleen-derived stromal cells express osteoblast-specific genes when combined with a proliferating factor (*FGF2*) and a differentiating hormone (dexamethasone) in the rat (Derubeis, Mastrogiacomo et al. 2003). The finding by Kodama *et al* that the spleen of adult mice contains a putative mesenchymal stem cell population through persistent expression of *Hox-11* supports this theory, whilst providing a possible future role for translational therapeutic strategies (Kodama, Davis et al. 2005). *Hox-11 / Tlx-1* +ve cells possess key stem cell characteristics by virtue of their capacity to self-renew and also to differentiate into cells of multiple lineages (references within Lonyai, Kodama et al. 2008). As previously mentioned (Chapter 2, section 2.4.1), this same group have also recently published evidence that the adult human spleen uniquely possesses a reservoir of multi-lineage adult stem cells that abundantly express *Hox-11 / Tlx-1* throughout adulthood (Dieguez-Acuna, Gygi et al. 2007). These may be an attractive cellular target for future translational research.

Alternatively, the process of iMET from splenic tissue could be attributed to a process of transdifferentiation. When the spleen is harvested for recombinations in the experiments described here, progenitor cells have already condensed to make the

spleen, adjacent to the stomach and dorsal pancreas. These differentiated cells may in fact transform into the pancreatic endocrine lineage when a specific milieu is created from recombining these spleens with pancreatic endocrine tissue. As previously mentioned (Chapter 2, section 2.5), mice bearing a null allele of the PTF1-p48 gene were born without an exocrine pancreas but harboured functional islets within the spleen (Krapp, Knofler et al. 1998). Although it is possible these cells were epithelial derived, one cannot rule out transdifferentiation of the splenic mesenchyme into endocrine cells as an explanation for their findings.

4.5 Conclusion

In conclusion, these data show that splenic mesenchyme is able to undergo iMET. This may be attributed to a possible stem cell population within the spleen or the spleen may be a responsive target for transdifferentiation. However, this iMET process does not occur in every recombinant and its frequency of occurrence may be altered by altering the tissue constituents within the recombinant. The molecular mechanisms behind this iMET process are investigated further in Section Three.

SECTION THREE

Determining the molecular mechanisms behind islet Mesenchyme-to-Epithelial Transition in the developing spleen

Chapter Five

Optimisation of an *in situ* hybridisation protocol for *Tlx-1* and characterisation of splenic *Tlx-1* expression during normal avian foregut development

5.1 Introduction

Tlx-1 (previously known as *Hox-11*) is an essential transcription factor for the specification of splenic fate during development (see Chapter 2, section 2.4.1), as shown by gene knockout studies (Dear, Colledge et al. 1995; Roberts, Sonder et al. 1995). Mouse embryo studies have found *Tlx-1* expression in the branchial arches, pharynx, heart, hindbrain, pinna, external auditory meatus and the spleen during development (Raju, Tang et al. 1993; Roberts, Shutter et al. 1994; Dear, Colledge et al. 1995; Logan, Wingate et al. 1998). It is known to be expressed in the embryonic mouse spleen from E11.5 to E13.5, with weaker expression from E14.5 onwards (Roberts, Shutter et al. 1994). Logan *et al* (1998) studied the expression of this gene in the cranial sensory ganglia and hindbrain in chick embryos and reported that it was also expressed in similar regions to that previously reported in the mouse, with prominent expression from stage 20 onwards in the splenic primordium. However, splenic *Tlx-1* expression during normal chick development has not yet been fully characterised. Expression is known to persist until birth in the mouse, and continues to be expressed post-natally in the adult mouse spleen (Kanzler and Dear 2001). Also, there is now evidence for continued expression of this early developmental marker in CD45 -ve cells of the adult human spleen, as previously mentioned in Chapter 2, section 2.4.1 (Kodama, Davis et al. 2005; Dieguez-Acuna, Gygi et al. 2007).

It was important at the beginning of this research project to have a splenic marker to confirm the splenic origin of the tissue being harvested. This gene would also be a useful splenic marker for the microdissected controls (to assess clean separation; see Chapter 4, Tables 2 and 3). In addition, it would be interesting to study the expression of this gene in the splenic mesenchyme when recombined with pancreatic epithelia in

recombinant chimaeric mini-organs. Firstly, however, the normal expression profile of *Tlx-1* in the avian spleen would need to be characterised before any assessment of its expression in chimaeric recombinant organs could be made. Therefore, the main aims of the experiments in this chapter were to: (i) establish a working *in situ* hybridisation protocol for *Tlx-1*, and (ii) characterise the normal avian splenic *Tlx-1* expression profile during our developmental period of interest (E4-E11).

5.2 Methods

5.2.1 Solutions and equipment

Solution formulae are shown in Appendix III and / or within the relevant subsections. Ultrapure distilled water (dH_20) was obtained from a Maxima USF Elga purification unit (resistivity 18.2 MΩ cm; USF Elga, Bucks, UK). To ensure absence of RNase activity, solutions and equipment used for *in situ hybridisation* were made up using RNase-free molecular biology grade water (ddH_20) purchased from vWR International (Leics, UK) and / or autoclaved for 20 minutes at 123°C before use (Benchtop Compact 40; Priorclave, Gt Lon, UK).

5.2.2 Synthesis of RNA probes for in situ hybridisation

The *Tlx-1* RNA probe was synthesised from a bluescript DNA plasmid containing the chick *Tlx-1* gene, a generous gift from Dr Cairine Logan, Department of Anatomy and Neuroscience Research Group, Faculty of Medicine, University of Calgary, Canada.

5.2.3 Transformation and amplification of chemically competent bacteria

Chemically competent, ampicillin resistant One Shot MAX Efficiency DH5α – T1R Escherichia coli (Invitrogen, Paisley, UK) were transformed with probe plasmids using a heat shock technique (30secs at 42°C), in accordance with the manufacturer instructions.

Transformed bacteria were then incubated overnight on Luria-Bertani (LB) agar plates containing 50mcg/ml 5-bromo-4-chloro-4-indolyl-beta-D-galactopyranoside (X-Gal) and 0.1% ampicillin in a humid environment containing 5% CO_2 and 95% air at 37°C. X-Gal is hydrolysed by bacterial beta-galactosidase to form an intense blue precipitate, allowing blue-white colony selection. The inclusion of ampicillin prevents incubation of contaminating bacteria. Individual blue colonies harbouring the plasmid were then selected and amplified by overnight incubations in 5ml and then 1000ml LB medium containing 0.1% ampicillin, shaking at 37°C.

5.2.4 Plasmid DNA purification and linearization

Amplified bacteria were lysed and the plasmid DNA was purified from the supernatant using a Plasmid Maxi Kit (QIAGEN, Crawley, UK) according to manufacturer instructions, available at:

http://www1.qiagen.com/literature/handbooks/PDF/PlasmidDNAPurification/PLS_Plasmid/1034637_HB_QIAGENPlasmid_112005.pdf

Purified plasmid DNA was then cleaned and concentrated using a MinElute Reaction Cleanup Kit (QIAGEN, Crawley, UK) according to manufacturer instructions, available at:

http://www1.qiagen.com/literature/handbooks/PDF/DNACleanupAndConcentration/MinElute/1027886_HB_QQ_MinElute_0604.pdf

Sequencing of the resultant DNA was performed to confirm correct alignment. Plasmid DNA nucleotide sequences were obtained from the DNA Sequencing Facility, Department of Biochemistry, University of Oxford. Alignment was assessed using the online NCBI nucleotide BLAST engine (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*), see Figure 13.

Plasmid DNA was linearised by plasmid-specific restriction enzymatic digestion. *Tlx-1* plasmid DNA was linearised by treatment with Xba-1 restriction enzyme (New England Biolabs, Herts, UK) buffered by NEBuffer 2 (New England Biolabs, Herts, UK) for 2 hours at 37°C. Linearised DNA then underwent an additional clean and concentration step as outlined previously. At the end of this MinElute Reaction Clean Up Kit protocol, the eluted DNA was re-centrifuged back through the column as an additional final step, in order to improve the yield.

5.2.5 RNA transcription

Linearised DNA was transcribed for 2 hours using digoxygenin (DIG)-UTP RNA labelling mix (Roche, West Sussex, UK) and T7 RNA polymerase (Roche, West Sussex, UK). Following this, the resultant RNA was cleaned and concentrated using an RNeasy MinElute Cleanup Kit (QIAGEN, Crawley, UK) according to manufacturer instructions, available at:

http://www1.qiagen.com/literature/handbooks/PDF/RNACleanupAndConcentration/RNY_MinElute_Cleanup/1023760_HBRNYME0303WW.pdf

Figure 13 Sequence alignment of *Tlx-1* plasmid

```
P'mid     67   CTTTTATTTCTCGTTTCCACATAAATTACACAAGCACTTTATAAAATGAATACACAGAAA  126
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24305   CTTTTATTTCTCGTTTCCACATAAATTACACAAGCACTTTATAAAATGAATACACAGAAA  24364

P'mid  127     ACACCTTATAAGTGCATAACTTAAAAAAAAAAAAAGCCTTAAAAAAATCTCACTGAAAATA  186
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24365   ACACCTTATAAGTGCATAACTTAAAAAAAAAAAAAGCCTTAAAAAAATCTCACTGAAAATA  24424

P'mid  187     GGTTTTCTGCTCGCTTGCCTGTACTAGCTGTTTAAACTGGCAGTGCTGTTTTAGATTATA  246
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24425   GGTTTTCTGCTCGCTTGCCTGTACTAGCTGTTTAAACTGGCAGTGCTGTTTTAGATTATA  24484

P'mid  247     AGTAACTGAAAAATATATACTGTATATATAATATCTATATATTCCCGGCGTTTGCTATAG  306
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24485   AGTAACTGAAAAATATATACTGTATATATAATATCTATATATTCCCGGCGTTTGCTATAG  24544

P'mid  307     GAAGTGCTTAATTGTGTCTCATTCCCTCAGAACAATTAAAGAAACAACAAAGTCGGAAGT  366
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24545   GAAGTGCTTAATTGTGTCTCATTCCCTCAGAACAATTAAAGAAACAACAAAGTCGGAAGT  24604

P'mid  367     GAAATCCATTTTCACCCTCGCAAACCTCCTGCCTCATCAAGGGACGCGGGTAGAGCAACC  426
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24605   GAAATCCATTTTCACCCTCGCAAACCTCCTGCCTCATCAAGGGACGCGGGTAGAGCAACC  24664

P'mid  427     CGTGGGGCCGCGCTTTGCAAATGATATGGAAATAACCCATGGAACGGAGCAGCAGCACCC  486
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24665   CGTGGGGCCGCGCTTTGCAAATGATATGGAAATAACCCATGGAACGGAGCAGCAGCACCC  24724

P'mid  487     CTGCCCAGCGCCGCCGGGCTCTCCTGGTTATTAAAGCTCGGGCTGATCGGCGCCGTGGCG  546
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24725   CTGCCCAGCGCCGCCGGGCTCTCCTGGTTATTAAAGCTCGGGCTGATCGGCGCCGTGGCG  24784

P'mid  547     GCCGGGGACAGTTTGGGCTCTTCGTGCAATTGAGATATCACATTTACCGTCCTCAGGAGG  606
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24785   GCCGGGGACAGTTTGGGCTCTTCGTGCAATTGAGATATCACATTTACCGTCCTCAGGAGG  24844

P'mid  607     CTGTCAGTTCCTTCGCTGCTTCCCCCCTCGCAGGAAAAAA-TTAATGAAAAAGGGGAGGA  665
               |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
G'nome 24845   CTGTCAGTTCCTTCGCTGCTTCCCCCCTCGCAGGAAAAAAATTAATGAAAAAGGGGAGGA  24904

P'mid  666     GACGCCGCTAGGTGAGGTCCGAAGGGCTGCCTGCTGCCGCCAGCACAAATTGCTGCTGGG  725
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24905   GACGCCGCTAGGTGAGGTCCGAAGGGCTGCCTGCTGCCGCCAGCACAAATTGCTGCTGGG  24964

P'mid  726     AGGCCAAGAGACGGGTGGCTTTCTCCATGTGTTGCGTCCCCAAACGTCTCCAGCGCCAAG  785
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 24965   AGGCCAAGAGACGGGTGGCTTTCTCCATGTGTTGCGTCCCCAAACGTCTCCAGCGCCAAG  25024

P'mid  786     GGCCCGGCGCGGTGCCTGGCTGCACAAACACGCTTCC-AAGGAGCCGTGTCCTTTTGTTC  844
               |||||||||||||||||||||||||||||||||||| ||||||||| ||||||||||||
G'nome 25025   GGCCCGGCGCGGTGCCTGGCTGCACAAACACGCTTCCCAAGGAGCCGCGTCCTTTTGTTC  25084

P'mid  845     CGATCAGTCCAAAGCGCGTACCGATAGACGCGCGTTATATATACATACAGTCCTACGGGG  904
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G'nome 25085   CGATCAGTCCAAAGCGCGTACCGATAGACGCGCGTTATATATACATACAGTCCTACGGGG  25144

P'mid  905     TGCCTCCAGCCCTGCAG  921
               |||||||||||||||||
G'nome 25145   TGCCTCCAGCCCTGCAG  25161
```

Fig 13 *Tlx-1* plasmid sequence alignment against the Gallus gallus chick genome, using the online NCBI nucleotide BLAST engine. The *Tlx-1* probe was a kind gift from Cairine Logan (1998, Journal of Neuroscience; 18:5389-5402), and consisted of an ~800 base pair sequence entirely contained within the 3' untranslated region of the gene (chromosome 6; NW_001471720). Alignment against the genome confirmed *Tlx-1* identity, with 99% homology to this region (identities= 854 / 857, GenBank accession number AF071874).P'mid = plasmid sequence, G'nome = untranslated region of *Tlx-1* from Gallus gallus genome.

5.2.6 Avian embryonic tissue procurement and dissection

Fertile chick (*Gallus domesticus*, hybrid Joice/Hill; Winter Egg Farm, Royston, Herts, UK) eggs were incubated at 37°C in a humid environment for the required gestation. Developmental stage of the embryos was determined according to Hamburger and Hamilton staging (Hamburger and Hamilton 1951) ; see also Appendix IV). The embryos were removed from the eggs and placed in chilled (4°C) Leibovitz's L-15 medium (Invitrogen, Paisley, UK). Intact abdominal organ blocks were then microdissected from the embryo, using a Leica MZ-16 dissecting microscope and electronically sharpened tungsten needles. All embryonic manipulation was performed under sterile conditions and in accordance with United Kingdom Home Office regulations under the Animals (Scientific Procedures) Act 1986. Abdominal organ blocks typically included the stomach, liver, pancreas, kidneys, spleen and foregut. These organ blocks were fixed for at least 2 hours using freshly-thawed phosphate-buffered 4% paraformaldehyde (PFA), with addition of EGTA 2mM. The fixed tissue was then washed in PBS before slowly dehydrating the tissue down a methanol series (25%, 50%, 75%, 100%) diluted in CMF-PBS, and stored at -20°C overnight, or until later required for *in situ* hybridisation

5.2.7 Whole-mount in situ hybridisation

Hybridisation was performed with digoxigenen (DIG) – labelled riboprobes (modified after Nieto *et al.*, 1996). Details of solutions are given in Appendix III: Supplemental solution information. Hybridisation experiments included positive and negative controls. Strict RNase-free conditions (gloves, RNase-free solutions etc) were

observed during the *in situ hybridisation*, especially on Day One of the protocol. The *in situ* hybridisation protocol was performed over a three (or four) day period:

Day One

The tissue stored in 100% methanol at -20°C were rehydrated back down the methanol series and washed twice in PBT (10 minutes for each wash). The tissues were prepared for hybridisation with 20 minutes incubation in 10µg/ml proteinase K in PBT, followed by two PBT washes (5minutes each). The tissue was subsequently fixed in 4% PFA with 0.2% glutaraldehyde for 20 minutes and washed twice in PBT (10 minutes each). The tissue was then washed for 10 minutes in prehybridisation buffer at 70 °C, before incubating in prehybridisation buffer for 2 hours at 70 °C. Following this, the tissue was incubated overnight in 1 µg / ml digoxigenen (DIG) labelled mRNA probe at 70 °C.

Day Two

Following overnight hybridisation, tissues were washed the next day at 70 °C in the following solutions (5 minutes for each wash): 100% Solution 1, then 75% Solution 1 : 25% 2x SSC (3M sodium chloride, 300mM tri-sodium citrate dihydrate), then 50% Solution 1 : 50% 2x SSC, then 25% Solution 1 : 75% 2x SSC. Following this, two 30 minute washes were carried out at 70°C in 2x SSC + 0.1% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) followed by two 30 minute washes at 70°C in 0.2x SSC + 0.1% CHAPS. Two 10 minute washes in TBTx at room temperature were performed followed by incubation in blocking solution for 2 – 3 hours (10% sheep serum, 2% Bovine Serum Albumin, in TBTx), before

incubating overnight in sheep anti-DIG alkaline phosphatase-conjugated antibody (1:200; Roche, West Sussex, UK) at 4°C.

Day Three

Following overnight incubation, the antibody was thoroughly washed off for at least ten washes (30 minutes each) at room temperature in TBTx containing 0.1% Bovine Serum Albumin. The tissue could optionally undergo further overnight washes in TBTx and 0.1% BSA (at 4°C), as additional washing off of the antibody helps to reduce "background" staining during the colour reaction. The tissue was then washed three times in AP buffer before incubating in the dark in a colour reaction solution containing NBT (nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl phosphate , toluidine salt). When the colour had developed (dark blue/indigo colour), colour visualisation was enhanced by transferring into fresh PBT, before fixation in fresh phosphate-buffered 4%PFA.

5.2.8 Bright-field microscopy

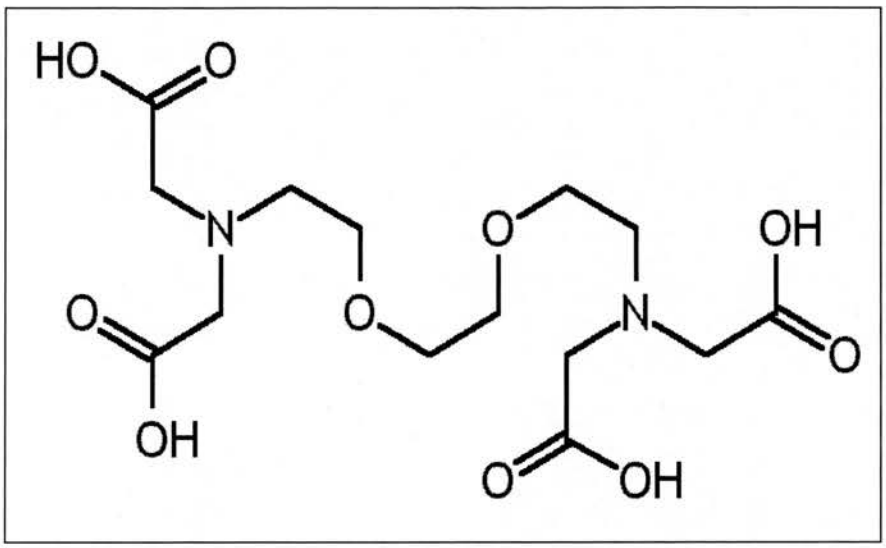
High-resolution (2560x1920 pixels) interpolated RGB images were captured using a Micropublisher 5MP RT digital camera mounted on a Zeiss Axioskop microscope

5.2.9 Tlx-1 expression during normal foregut development

Materials and methods were all as previously described. Chick embryos were harvested for embryonic days 4 to 11 (Hamburger-Hamilton stages 23 to 37) and *Tlx-*

1 in situ hybridisations were performed on their abdominal organ blocks. Following this, abdominal organ blocks were sectioned (12μm) for each developmental day.

Figure 14 Chemical structure of EGTA



5.3 Optimisation of a protocol for *Tlx-1 in situ* hybridisation

The successful *in situ* hybridisation protocol described in section 5.2.7 is a modified protocol from that previously used in this laboratory. The previous unmodified laboratory protocol had successfully been used with other probes (*Shh*, *Barx1*, *Pdx1*) and so was also initially performed for the *Tlx-1* probe on E3-E8 chick and quail embryos. However, although expression in other *Tlx-1* expressing regions was present (pharyngeal arches, heart etc.), splenic expression was very weak and variable. Initially this was thought to be due to poor penetration of the probe through the body wall to reach the spleen. This was addressed by dissecting out the abdominal organ block from the embryo and performing the *in situ* hybridisation on this excised tissue. However, splenic expression remained poor and so the *in situ* was repeated using freshly re-transcribed *Tlx-1* (transcribed RNA was confirmed adequate using gel electrophoresis and measuring the Optical Density using spectrophotometry) and using freshly made solutions, but again with only weak expression and only on the periphery of the spleen, but strong gut endodermal *Shh* positive control expression (Apelqvist, Ahlgren et al. 1997). Therefore, transformation of new DH5 competent cells with the *Tlx-1* plasmid was undertaken, followed by amplification and linearization to produce fresh *Tlx-1* DNA, before transcribing fresh *Tlx-1* RNA probe (DNA or RNA produced at each stage was again confirmed adequate by gel electrophoresis and spectrophotometry). The *Tlx-1* plasmid sequence was correctly aligned, confirming that this was the correct sequence for *Tlx-1* (see Figure 13). However, repeat *in situ* hybridisation with this probe was again very poor, but strong distinct positive control expression in the stomach was seen using the stomach mesenchymal marker *Barx-1* (Kim, Buchner et al. 2005).

The *in situ* hybridisation protocol itself was then modified, incorporating additional steps from *in situ* hybridisation protocols in the literature (Wilkinson 1992; Streit and Stern 2001; Hargrave, Bowles et al. 2006). This produced excellent distinct splenic staining, indicating that the degree of success with this probe was dependent on the type of protocol used. This modified protocol involved the ubiquitous addition of Ethylene Glycol Tetra-acetic Acid (EGTA; see Figure 14) to all 4% PFA fixative used. In addition, 0.2% glutaraldehyde was also added to the fixative used on Day One. 5% Poly Vinyl Alcohol (PVA) was initially also added to the AP buffer on Day Three of the protocol. However, the *in situ* worked as clearly without PVA as it did with, and so this was subsequently omitted from the final modified *in situ* hybridisation protocol. Different post-harvesting fixation periods were also finally compared (1 hour, 2 hours, 6 hours and 24 hours), as this has been reported as a crucial factor for the quality of the final *in situ* hybridisation signal (Streit and Stern 2001). However, no difference was observed with different fixation times, with either *Tlx-1* or *Barx-1 in situ* hybridisations (all *in situ*'s performed equally well with different fixation periods).

This new successful modified *in situ* hybridisation protocol is detailed in the previous section (Section 5.2.7). This protocol also worked very well with other probes used in our laboratory for *in situ* hybridisation reactions (including *Pdx-1* and *Barx-1*) and is now the preferred protocol for *in situ* hybridisations in our laboratory.

5.4 *Tlx-1* expression during normal avian foregut development

Tlx-1 was expressed in the spleen of all abdominal organ blocks at each developmental stage analysed (see Table 5 and Figure 15). It was only expressed in the spleen, with no expression observed in other abdominal viscera or surrounding tissues. Interestingly, the spleen begins in a caudal position in relation to the stomach until E6 but then gradually assumes an increasingly rostral position relative to the stomach, until it achieves a position cranial to the stomach by E9.

Table 5 Splenic *Tlx-1* expression for each embryonic stage

Day of gestation	Embryonic stage (HH)	Sample number	Splenic *Tlx-1* expression
4	23/24	5	+
5	27	4	+
6	28/29	8	+
7	30	6	+
8	34	6	+
9	35	9	+
10	36	8	+
11	37	6	+

Figure 15 Chick embryo abdominal organ blocks after *Tlx-1 in situ* hybridisation

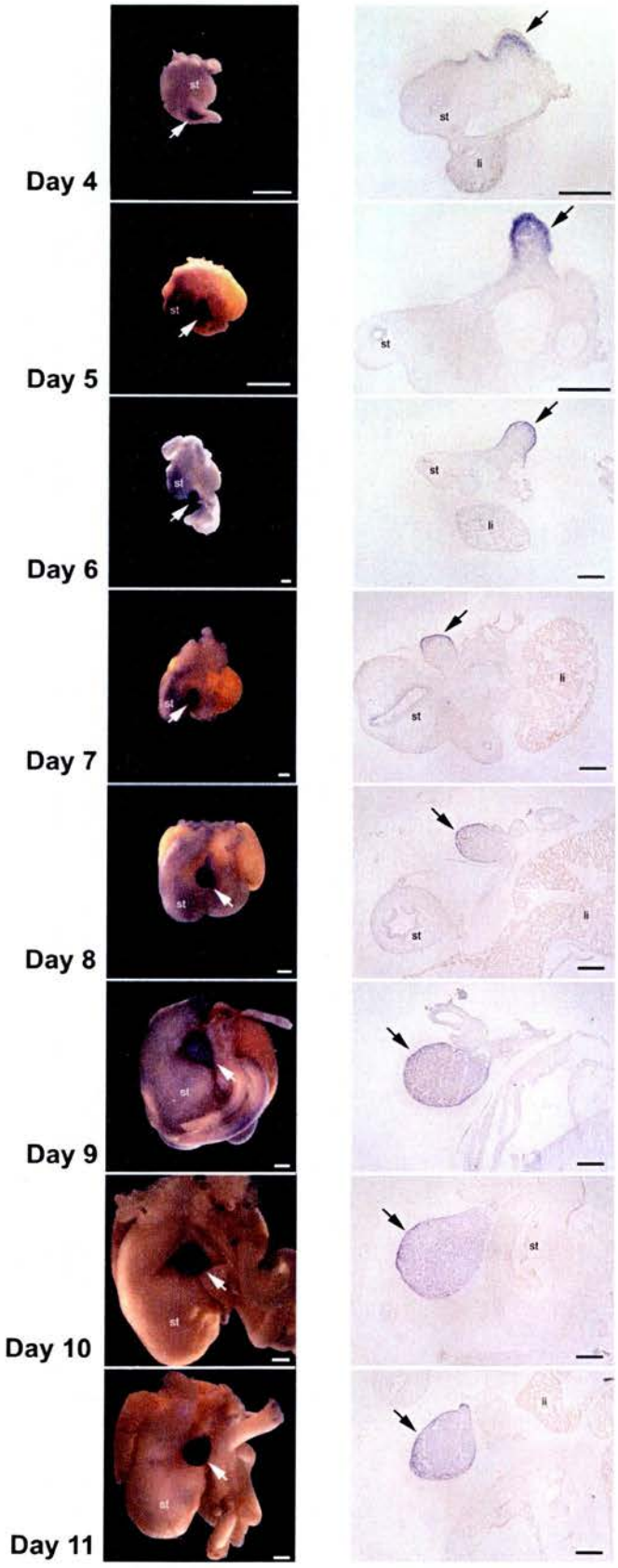


Fig 15

Left panels (black background): Dorsal whole-mount view of microdissected chick embryo abdominal organ blocks after *Tlx-1 in situ* hybridisation on the indicated days of development. The spleen is stained in blue in each image (also identified by arrow), in close proximity to adjacent stomach (st).
Scale bars 1mm.

Right panels (light background): Cross-sections (12µm) through chick embryo abdominal organ blocks after *Tlx-1 in situ* hybridisation for each indicated day of development; the spleen is stained in blue (identified by arrow), st=stomach; li=liver.
Scale bars 500µm.

5.5 Discussion

The *Tlx-1 in situ* hybridisation protocol was successfully optimised through two combined protocol modifications; the ubiquitous addition of EGTA to the fixative and addition of glutaraldehyde to the fixative on Day One of the protocol.

EGTA is a calcium chelator which specifically binds Ca^{2+} ions. This is in comparison to another chelator Ethylene Diamine Tetraacetic Acid (EDTA), which chelates a wide range of divalent cations but with less affinity specifically for calcium. EGTA also stabilises the pH. Historically, EGTA has been widely used in fixatives as it was thought to help cytoskeletal stabilisation. The cytoskeleton is very dynamic and sensitive to changes in the chemical and mechanical environment. EGTA helps to inhibit enzymes and so halts cellular activity, thus preserving the cellular structure and so optimising the *in situ* hybridisation signal. EGTA's enzyme-inhibiting effect may also inhibit RNase activity, thus improving the *in situ* hybridisation signal. Interestingly, EGTA has previously been reported to inhibit nucleases (Wang and Gegenheimer 1990) and there has been a report that RNase activity is Ca^{2+} dependent (Gbenle and Akinrimisi 1984). Decalcification has also been previously reported to improve the sensitivity of fluorescent *in situ* hybridisation techniques using EDTA (Brown, Edwards et al. 2002; Martinez-Ramirez, Cigudosa et al. 2004; Korac, Jones et al. 2005), through preservation of the DNA (Brown, Edwards et al. 2002). An alternative explanation for the improved *in situ* hybridisation signal could be that removing calcium from the cell membrane permits increased pore size formation (during the Proteinase-K step), thus improving probe access to the mRNA. However, EGTA may have some detrimental effects when used to fix certain plant tissues

(Wang, Liu et al. 2005), which may partly explain why it has not continued to be so widely used for *in situ* protocols.

Glutaraldehyde is a fixative which helps to preserve the cell structure. It forms cross-links with proteins in close proximity to help protect the mRNA. It has been reported to improve detection of gene expression when used in the fixation step (Ma, Rogers et al. 2002), and has been used as a fixative in past *in situ* hybridisation protocols in the literature (Godard and Jones 1980; Paeratakul, De Stasio et al. 1988; White and Gorovsky 1988). However, its use may have declined owing to its potentially harmful toxic properties.

Optimal tissue fixation is known to be important in gene expression studies and results can vary according to variations in the fixative type, temperature and duration of fixation (Streit and Stern 2001; Ma, Rogers et al. 2002). Interestingly, no difference was observed in the quality of the final *in situ* hybridisation signal with different fixation times in these experiments.

PVA has been reported to enhance the detection of low abundance transcripts in quail embryos during *in situ* hybridisation reactions (Barth and Ivarie 1994). However, we found the addition of this to be unnecessary, as we discovered that the protocol continued to work excellently when this agent was omitted.

Tlx-1 is normally expressed within the developing chick spleen throughout the developmental period studied (E4-E11). This is in keeping with a previous study in the literature describing its expression in the mouse (Dear, Colledge et al. 1995;

Kanzler and Dear 2001). It is also known to be expressed in other regions of the embryo, such as the branchial arches, pharynx, heart, hindbrain, pinna and external auditory meatus (references in Chapter 2, section 2.4.1). However, there is no expression in other abdominal viscera or surrounding tissues, and this is confirmed in this study. This makes it a very useful spleen-specific developmental marker for use in this thesis. Now that *Tlx-1* expression has been characterised during normal splenic development, the regulation of this gene can be studied in the splenic mesenchyme of chimaeric recombinant organs, together with genes responsible for islet development.

5.6 Conclusions

The *Tlx-1 in situ* hybridisation reaction was successfully optimised through modifications to the protocol. This involved the ubiquitous addition of EGTA to all fixative used, plus addition of glutaraldehyde to the fixative on Day One of the *in situ* protocol.

Tlx-1 is normally expressed within the developing chick spleen throughout the developmental period studied (E4-E11). There is no expression in other abdominal viscera or surrounding tissues, making it a very useful spleen-specific developmental marker for use in this thesis. Now that splenic *Tlx-1* expression has been characterised during normal development, the regulation of this gene can be studied in the splenic mesenchyme of chimaeric recombinant organs, together with genes responsible for islet development. This is investigated in the following chapters.

Chapter Six

Tlx-1 regulation in the splenic mesenchyme during islet Mesenchyme-to-Epithelial Transition

6.1 Introduction

An understanding of the molecular mechanisms underlying islet MET in the spleen would be of scientific interest on its own merit, but is clearly crucial if the potential for use in future therapeutic strategies for Diabetes Mellitus is to be harnessed. It is becoming increasingly evident that in order to persuade pluripotent cells to undergo islet cell differentiation, carefully coordinated provision of islet developmental cues are required (Miyazaki, Yamato et al. 2004; Brolen, Heins et al. 2005; Santana, Ensenat-Waser et al. 2006; Treff, Vincent et al. 2006; Roche, Ensenat-Waser et al. 2007; Saitoh, Yamato et al. 2007; Vaca, Berna et al. 2008). To inform the design of future differentiation strategies, the aim of this chapter (and subsequent chapters) was to examine the molecular mechanisms behind iMET. This was also important in understanding why splenic insulin expression was not observed in all recombinants, but whether there might be a spectrum of splenic tissue reprogramming, with insulin expression only being observed when the tissue had been fully reprogrammed.

The aim of this chapter was to investigate whether the key splenic transcription factor *Tlx-1* (see Chapter 5) continued to be expressed by the splenic mesenchyme within chimaeric recombinant organs, or whether the splenic mesenchyme was reprogrammed when recombined with pancreatic epithelium. The following hypothesis was generated: *Tlx-1 is down-regulated in the splenic mesenchyme of chimaeric recombinant organs*. Interest in this idea arose from previous investigation into avian pancreatogenesis in this laboratory, which found a down-regulation of the stomach mesenchymal transcription factor *Barx-1* when stomach mesenchyme was recombined with pancreatic epithelium (unpublished). In addition, previous attempts

to recapitulate renal MET have shown that mesenchymal stem cells are only competent to differentiate into renal epithelium when reprogrammed by signals from the differentiating epithelial ureteric bud (Yokoo, Ohashi et al. 2005).

6.2 Methods

Recombinants were generated as detailed previously (2 quail spleens recombined with 2 chick pancreatic epithelia) and cultured for 7 days before fixation. Microdissected quail spleen controls were fixed either at day 0 or day 1 of *in vitro* culture, since previous experience had found that mesenchymal cells cultured alone (in the absence of epithelium) do not tolerate *in vitro* conditions well (and certainly not surviving much beyond one day).

Following *in vitro* culture, wholemount *in situ* hybridisation for *Tlx-1* was performed as previously described, but with the addition of a 30 minute incubation of the tissue in 6% Hydrogen Peroxide in PBT, followed by two 5 minute washes in PBT, before the proteinase K step (on Day One of the protocol). This step was added to quench endogenous peroxidase activity.

Following the *in situ* hybridisation, the tissue underwent further fixation (limited to one hour). Fixative was washed off with PBS before incubating overnight with 30% sucrose in PBS. The tissue was then frozen in Tissue-Tek OCT compound (Sakura Finetek, Thatcham, UK), sectioned (10 microns thickness) and placed on polysine-coated microscope slides (vWR International, Leics, UK) ready for immunocytochemistry with a QCPN peroxidase reaction. Controls also underwent

wholemount *in situ* hybridisation for *Tlx-1* before further fixation, sectioning and analysis. Since the splenic tissue was all quail in origin, it was not necessary to routinely perform QCPN peroxidase reactions on these.

6.2.1 QCPN peroxidase reaction protocol

Following sectioning, the slides were washed in PBS then PBT before incubation in blocking buffer (as described previously) for 60 minutes. The slides were then incubated in QCPN primary antibody in blocking buffer (1:5) overnight. The antibody was washed off the next day. After this, a modified VECTASTAIN Elite Mouse IgG ABC kit protocol (Vector Laboratories, Peterborough, UK) was used: The primary antibody was washed off with PBS and then the slides were incubated in diluted biotinylated secondary antibody solution for 30 minutes. The secondary antibody was washed off in PBS multiple times before incubating for a further 30 minutes in VECTASTAIN Elite ABC reagent. The slides then underwent multiple washes in PBS before using a DAB (3,3-diaminobenzidine) Substrate Kit for Peroxidase (Vector Laboratories, Peterborough, UK) to perform the peroxidase substrate solution incubation. Only approximately 10 seconds peroxidase incubation were required for the brown stain intensity to occur. The peroxidase solution was then promptly washed off in tap water (as it contains nickel, which improves the stain). Slides were mounted in DPX (distyrene plasticiser xylene mixture) mountant before inspecting under a bright-field microscope.

All chimaeric recombinant mini-organs and controls were analysed using bright-field microscopy for antigen expression, interpreted together with morphological criteria.

Tlx-1 expression was analysed by recording whether the QCPN +ve regions (brown) had *Tlx-1* +ve expression (blue) or not, and QCPN regions could then be classified as either *Tlx1* +ve or –ve, or a "mixed" region if there was mixture of *Tlx-1* +ve and *Tlx-1* –ve areas within the QCPN +ve region.

6.3 *Tlx-1* is down-regulated in splenic mesenchyme during iMET

Nine spleen recombinants were produced (two quail spleens recombined with two chick pancreatic epithelia) and eleven quail spleen controls (six were fixed at day 0 *in vitro* culture and five underwent 1 day culture before fixation). All the controls were positive for *Tlx-1* (see Figures 16(b) and 16(c)). In addition, 8 quail abdominal organ blocks underwent simultaneous *Tlx-1 in situ* hybridisation (as a positive and negative control) and showed distinct positive splenic expression, with negative expression in all other regions of the abdominal organ block. No *Tlx-1* expression was detected in any of the recombinants (see Table 6), showing that the splenic mesenchyme in the recombinants had undergone dramatic 100% down-regulation of *Tlx-1* (see Figure 16(a)).

Figure 16 Comparison of *Tlx-1* expression in a splenic recombinant and spleen controls

Fig 16(a)

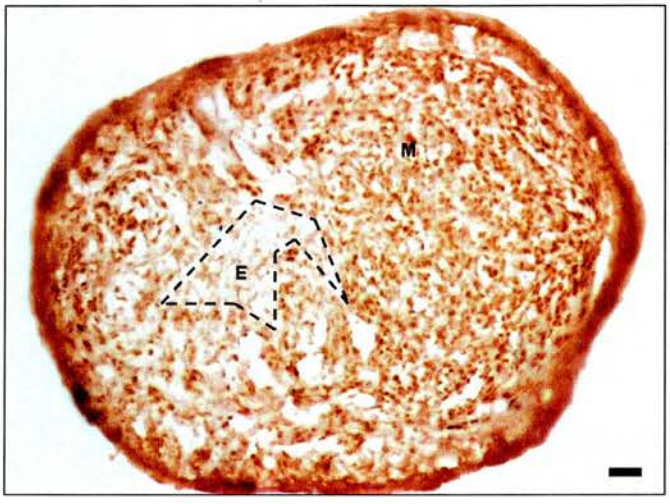


Fig 16(a) No blue *Tlx-1* expression seen in the splenic mesenchyme within a recombinant. M = brown QCPN nucleoli (splenic tissue); E = pancreatic epithelium; the super-imposed dashed line delineates the boundary between epithelium and mesenchyme in this section; 10μm section thickness, scale bar 10μm.

Fig16(b)

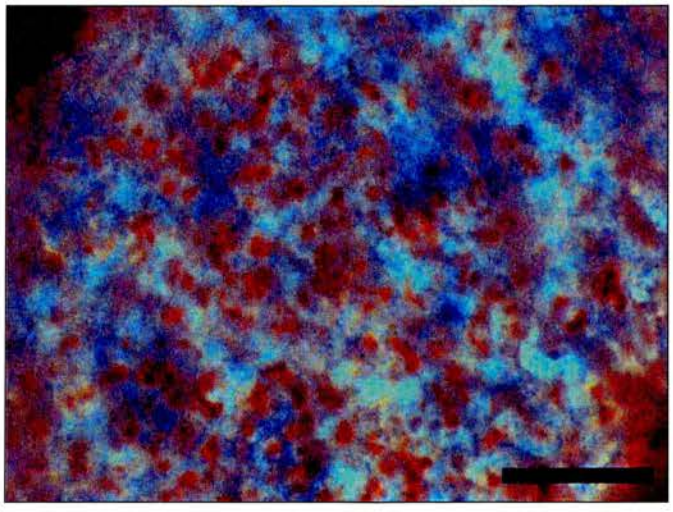


Fig 16(b) An example of combined *Tlx-1 and* QCPN expression in a splenic mesenchyme control (high power magnification). *Tlx-1 in situ* hybridisation is shown in blue and QCPN in brown. The *in situ* hybridisation was performed alongside the recombinant *in situ* hybridisation as a positive control. 10μm section thickness, scale bar 10μm.

Fig 16(c)

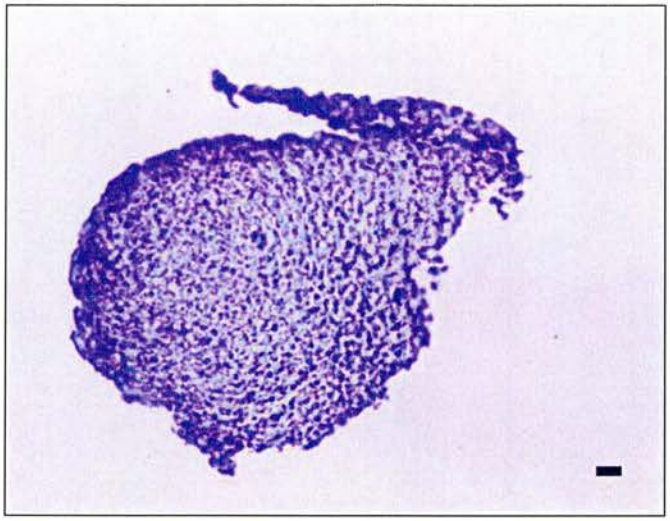


Fig 16(c) *Tlx-1* expression seen in a splenic mesenchyme control. The splenic tissue was all of quail origin and so a QCPN peroxidase step was not routinely required in the controls. This image shows a positive control for the *Tlx-1 in situ* hybridisation and was performed alongside the recombinant *in situ* hybridisation reaction. 10μm section thickness, scale bar 10μm.

Table 6 *Tlx-1* expression in each splenic recombinant

Recombinant	QCPN +, *Tlx1* - regions	QCPN +, *Tlx1* + regions	QCPN + regions, with some *Tlx1* + area ("mixed")
1	+	-	-
2	+	-	-
3	+	-	-
4	+	-	-
5	+	-	-
6	+	-	-
7	+	-	-
8	+	-	-
9	+	-	-

Table 6 showing which splenic recombinants had regions of combined *Tlx-1* +ve and QCPN +ve expression. No combined expression was detected, demonstrating dramatic down-regulation of this gene in the splenic mesenchyme.

6.4 Discussion

There was dramatic down-regulation (100%) of *Tlx-1* in all the recombinants in this series. We would normally expect splenic mesenchyme to express *Tlx-1* at this stage of gestation *in vivo* (E11; see Chapter 5, Figure 15). Therefore, the complete down-regulation of this transcription factor is due to recombining the mesenchyme with the pancreatic epithelium and is most likely to be mediated via signals received from the differentiating pancreatic epithelium. It is unlikely to be an epiphenomenon arising from tissue manipulation or culture conditions, although recombination of splenic mesenchyme with non-pancreatic epithelia would need to be performed to confidently exclude this. It would also be helpful to perform a viability assay on the recombinant tissue following the roller culture as a further additional control study to ensure the apparent 100% down-regulation was not due to the cells being damaged during the culture period. However, this would seem unlikely given that insulin, *pdx-1* and *isl-1* were all seen to be expressed by the cells at the end of the same culture period (see Chapters 4, 7 and 8). It is interesting to note that previous experiments in this laboratory studying *Barx-1* down-regulation in stomach mesenchyme, when recombined with either pancreatic epithelia or stomach epithelia, have shown that the mesenchyme is reprogrammed specifically through recombination with pancreatic epithelia (unpublished data). It is very likely that the same principle applies with splenic mesenchyme and that recombination of the splenic mesenchyme with another tissue (in this case pancreatic epithelium) is required to re-program the mesenchyme to no longer express the splenic transcription factor *Tlx-1*.

Tlx-1 gene knockout studies show that expression of this gene is essential for splenic development, possibly via increased proliferation of mesenchymal cells or reduced apoptosis (see Chapter 2, section 2.4.1). The role of *Tlx-1* in proliferation is consistent with reports that *Hox-11* +ve human acute lymphoblastic leukaemia (ALL) is caused by a translocation on chromosome 10. This translation breaks-off *Hox-11* from upstream regulation, resulting in uncontrolled proliferation of *Hox-11* target genes (Dube, Kamel-Reid et al. 1991; Hatano, Roberts et al. 1991; Kennedy, Gonzalez-Sarmiento et al. 1991). Taken together, this evidence suggests that *Tlx-1* has a proliferative role (Lonyai, Kodama et al. 2008). This stem-cell like property may mean that cells expressing this gene are amenable to manipulation for regenerative cellular therapies. As previously mentioned (Chapter 2, section 2.4.1), there is also evidence for continued expression of this early developmental marker in CD45 -ve cells of the normal adult human spleen (Kodama, Davis et al. 2005; Dieguez-Acuna, Gygi et al. 2007), which may be useful in future therapeutic strategies if such a cell source can be manipulated using human splenic tissue. A promising and ethical future strategy might be to use mature *Tlx*-1 expressing splenic tissue from the diabetic patient for autologous transplant (following *in vitro* manipulation into islets) to treat their diabetes, which may limit or reduce the need for immunosuppression currently required for an allograft (Kodama, Davis et al. 2005; Robertson, Rowan-Hull et al. 2008). This would need to be balanced against any potential safety risks with this gene, given its association with ALL. However, *Tlx-1* is known to be expressed in normal adult human spleens and the tumorigenesis in ALL is due to translocation from its upstream regulatory genes. Furthermore, the experiments in this chapter indicate that this gene is completely down-regulated during iMET, providing a further degree of safety.

6.5 Conclusions

The results suggest that *Tlx-1 is down-regulated in splenic mesenchyme when recombined with pancreatic epithelia*. This is a complete down-regulation (100%) and indicates that the splenic mesenchyme is being genetically *reprogrammed* into an alternative non-splenic fate. The next two chapters investigate whether the pancreatic transcription factors *Pdx-1* and *Isl-1* are up-regulated in this tissue.

Chapter Seven

Pdx-1 regulation in the splenic mesenchyme during islet Mesenchyme-to-Epithelial Transition

7.1 Introduction

The aim of this chapter was to investigate whether the homeodomain transcription factor *Pdx-1* was expressed by the splenic tissue within chimaeric recombinant organs. *Pdx-1* expression is extremely important for pancreatic organogenesis and is considered the pancreatic "master gene". It is expressed by all pancreatic epithelial precursors and null expression results in a failure of pancreatic development in mice (Jonsson, Carlsson et al. 1994). During mouse development, *Pdx-1* expression begins at E8 (Ohlsson, Karlsson et al. 1993; Guz, Montminy et al. 1995; Li, Arber et al. 1999), before the onset of pancreatic bud formation and islet hormone gene expression. *Pdx-1* is initially expressed broadly in the primitive posterior foregut, with pancreas development being initiated only in a restricted region of *Pdx-1* expressing posterior foregut in which endodermal *Shh* expression is repressed. Interestingly, when *Shh* is artificially inhibited with a *Shh* blocker, pancreatic expansion is promoted but also with ectopic pancreatic endocrine and exocrine structures developing in regions adjacent to the pancreas, such as the stomach and duodenum (Kim and Melton 1998). Once the pancreas has differentiated, *Pdx-1* expression is restricted to beta-cells, where it transactivates the insulin promoter (Ohlsson, Karlsson et al. 1993). *Pdx-1* is not expressed in foregut mesenchyme (Ahlgren, Jonsson et al. 1996). It was therefore important to determine whether the splenic mesenchyme was being reprogrammed to express this pancreatic gene in our chimaeric recombinants. This chapter addresses the hypothesis that *Pdx-1 is up-regulated in the splenic mesenchyme of chimaeric recombinant organs.*

7.2 Methods

Recombinants were generated as detailed previously (2 quail spleens recombined with 2 chick pancreatic epithelia) and cultured for 7 days before fixation. The previously described wholemount *in situ* hybridisation was performed using a *Pdx-1* probe, with the addition of a 30 minute incubation of the tissue in 6% Hydrogen Peroxide in PBT, followed by two 5 minute washes in PBT, before the proteinase K step (on Day One of the protocol). This step was added to quench endogenous peroxidase activity. The *Pdx-1* RNA probe was synthesised from a bluescript DNA plasmid containing the chick *Pdx-1* gene, a generous gift from Dr A. Grapin-Botton, Swiss Institute for Experimental Cancer Research (ISERC), Lausanne, Switzerland. The methodology was the same as that detailed previously, with the following exceptions during the generation of the *Pdx-1* probe; (i) The *Pdx-1* plasmid DNA was linearised by treatment with HindIII restriction enzyme (New England Biolabs, Herts, UK) buffered by NEBuffer 2 (New England Biolabs, Herts, UK) for 2 hours at 37°C; (ii) During RNA transcription, linearised DNA was transcribed for 2 hours using digoxygenin (DIG)-UTP RNA labelling mix (Roche, West Sussex, UK) with T3 (rather than the T7 used for *Tlx-1*).

Following the *in situ* hybridisation, the tissue underwent further fixation (limited to one hour). Fixative was washed off with PBS before incubating overnight with 30% sucrose in PBS. The tissue was frozen in Tissue-Tek OCT compound (Sakura Finetek, Thatcham, UK), sectioned (10 microns thickness) and placed on polysine-coated microscope slides (vWR International, Leics, UK) ready for immunocytochemistry with a QCPN peroxidase reaction. The QCPN peroxidase reaction was performed as

previously described. Controls also underwent wholemount *in situ* hybridisation for *Pdx-1* before further fixation, sectioning and analysis.

All chimaeric recombinant mini-organs and controls were analysed using bright-field microscopy for antigen expression, interpreted together with morphological criteria. The expression of *Pdx-1* was analysed by recording the blue *Pdx-1* positive regions within each recombinant and also whether there was co-expression of QCPN within the patch. The *Pdx-1* regions could then be classified as either QCPN +ve or QCPN – ve, or "mixed" if there was some epithelial and some QCPN +ve contribution to that *Pdx-1* region.

7.3 *Pdx-1* is up-regulated in splenic mesenchyme during iMET

5 / 9 (56%) spleen recombinants had regions of co-localisation of QCPN and *Pdx-1* indicating up-regulation of *Pdx-1* in splenic mesenchyme in these recombinants (see Figure 17 and Table 7). Nearly all the recombinants (8 / 9) had regions of QCPN negative *Pdx-1* positive expression, as would be expected from the pancreatic epithelium in the recombinant. Eleven quail spleen controls were analysed for *Pdx-1* (five were fixed at day 0 *in vitro* culture and six underwent 1 day culture before fixation). All these quail spleen controls were negative for *Pdx-1*, confirming clean separation (no quail pancreas contamination). In addition, 8 quail abdominal organ blocks also underwent simultaneous *Pdx-1 in situ* hybridisation (as a positive and negative control) and showed distinct positive pancreatic expression, with negative expression in all other regions of the abdominal organ block (see Figure 18 showing pancreatic *Pdx-1* expression in a wholemount E10 organ block control).

Figure 17 Sectioned image showing an example of a combined QCPN +ve *Pdx-1* +ve region in a splenic recombinant

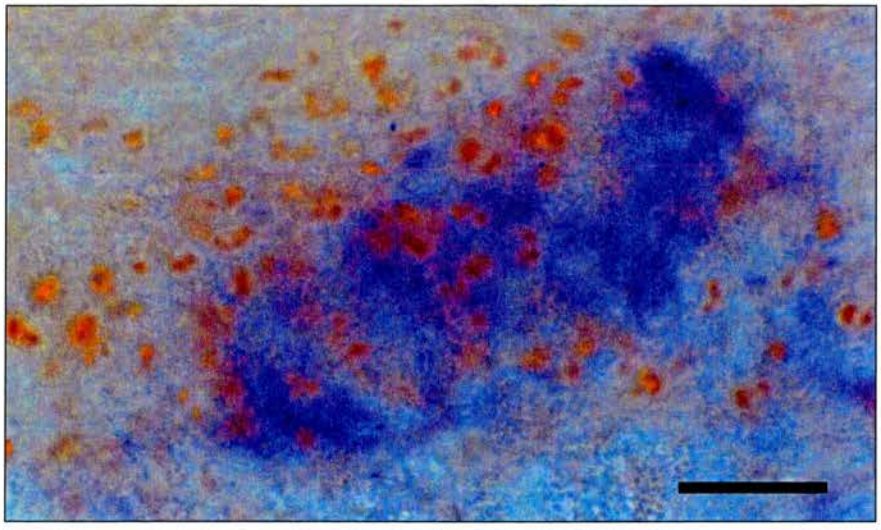


Fig 17 showing an area of splenic mesenchyme (shown by the brown QCPN nucleoli) expressing *Pdx-1* (in blue); 10μm section thickness, 10μm scale bar.

Figure 18 Chick pancreatic *Pdx-1* expression during normal development

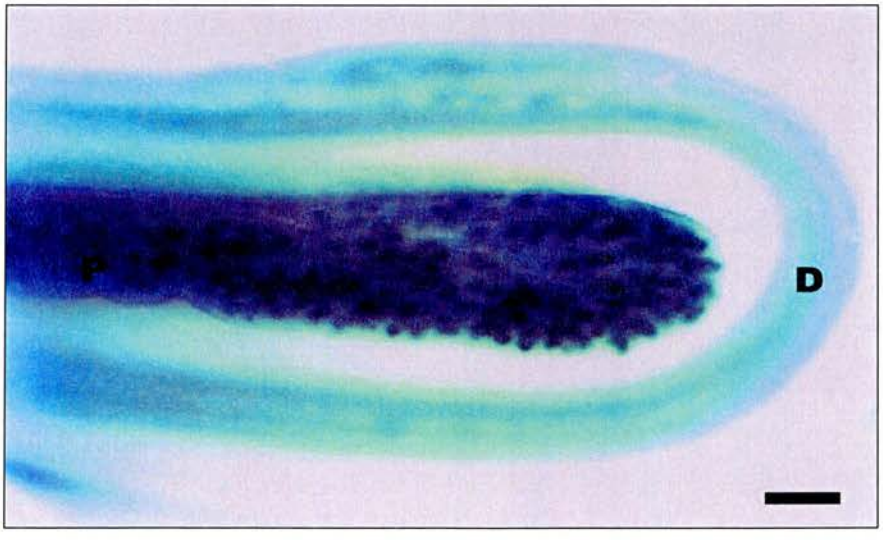


Fig 18 *Pdx-1* expression in the pancreas at day 10 of normal gestation (HH36). Distinct blue *Pdx-1* expressing Islets of Langerhans can be seen within the pancreas. Scale bar 100μm; P = pancreas, D = duodenum.

Table 7 *Pdx-1* expression in each splenic recombinant

Recombinant	QCPN -ve (epithelial) *Pdx-1* +ve	"Mixed" contribution (*Pdx-1* +ve region with some QCPN +ve areas)	QCPN +ve (splenic) *Pdx-1* +ve
1	+ (1)	+ (1)	+ (2)
2	-	-	+ (2)
3	+ (1)	+ (1)	-
4	+ (2)	+ (1)	-
5	+ (1)	-	-
6	+ (1)	-	-
7	+ (2)	+ (1)	-
8	+ (2)	-	-
9	+ (3)	-	-

Table 7 This table shows which splenic recombinants had combined *Pdx-1* +ve and QCPN +ve regions (number of regions indicated in brackets). Nearly all recombinants had *Pdx-1* +ve QCPN –ve regions, as expected from the pancreatic epithelia. However, 5 / 9 recombinants displayed regions of combined *Pdx-1* +ve QCPN +ve regions (including *Pdx-1* regions that were purely QCPN +ve, or of a "mixed" contribution with some QCPN +ve areas within it). This demonstrates up-regulation of this gene in the splenic mesenchyme.

7.4 Discussion

These results show that *Pdx-1 is up-regulated in the splenic mesenchyme when recombined with pancreatic epithelia* and this was observed in over a half of cases (56%). Table 7 shows that in these 5 recombinants, there was a *Pdx-1* region of "mixed" origin (with some QCPN +ve splenic contribution) in at least 3 recombinants whilst two recombinants contained *Pdx-1* regions of completely splenic origin (QCPN +ve, *Pdx-1* +ve). 8/9 recombinants contained *Pdx-1* regions of epithelial origin, as we would expect from the chick pancreatic epithelia. However, one recombinant contained no epithelial contribution, but only splenic *Pdx-1* regions (recombinant 2).

The *Pdx-1* –ve splenic controls give additional confidence over the safety of the dissection margins in this thesis. Whilst insulin is a useful indicator of pancreatic tissue, *Pdx-1* is more widely expressed by pancreatic tissue during early development and is expressed from an earlier gestation.

These distinct confluent *Pdx-1* +ve regions seen in the splenic mesenchyme of the recombinants (see Figure 17) presumably represent B-islets at this stage of gestation. Although the pancreatic epithelia is now day 11 gestation at the end of the *in vitro* culture period, it is difficult to accurately assess which day of pancreatic gestation the splenic tissue might be, as they were initially developing down a splenic fate (at day 4 gestation) until recombined with pancreatic epithelia. However, we know from the insulin studies (Chapter 4) that distinct insulin-expressing clusters, with islet morphology, have developed in the splenic mesenchymal tissue by this same stage, and so it is reasonable to suspect that these *Pdx-1* regions are islets too. As already mentioned, *Pdx-1* is expressed by all pancreatic epithelial precursors (Jonsson, Carlsson et al. 1994) and is later restricted to beta-cells (Ohlsson, Karlsson et al.

1993). Therefore, the *Pdx-1* up-regulation in the splenic mesenchyme at this stage indicates that this tissue is being reprogrammed into a pancreatic epithelial endocrine fate (ie: islet Mesenchyme-to-Epithelial Transition).

This finding of *Pdx-1* expression during splenic iMET is important because *Pdx-1* expression is known to continue in mature adult beta-cells (Ohlsson, Karlsson et al. 1993) and is essential for their function (Ahlgren, Jonsson et al. 1998; Holland, Hale et al. 2002). Loss of *Pdx-1* expression in mature beta-cells causes a dramatic decrease in their expression of insulin, *Nkx6.1* and the glucose transporter GLUT2 (glucose transporter type 2, which is a marker of mature beta-cells) (Pang, Mukonoweshuro et al. 1994; Ahlgren, Jonsson et al. 1998). Furthermore, mice heterozygous for a *Pdx-1* deficiency are glucose-intolerant (Ahlgren, Jonsson et al. 1998), consistent with the finding that humans carrying dominant *Pdx-1* mutations are predisposed to "maturity-onset diabetes of the young" (MODY), a form of T2DM (Stoffers, Ferrer et al. 1997; Macfarlane, Frayling et al. 2000).

7.5 Conclusions

These results show that *Pdx-1 is up-regulated in the splenic mesenchyme when recombined with pancreatic epithelia* and this was observed in over a half of cases (56%). *Pdx-1* up-regulation in the splenic mesenchyme indicates that this tissue can be reprogrammed into a pancreatic epithelial endocrine fate *in vitro* when recombined with pancreatic epithelium. The next chapter investigates whether *Isl-1* is also up-regulated in the splenic mesenchyme of recombinants.

Chapter Eight

Isl-1 regulation in the splenic mesenchyme during islet Mesenchyme-to-Epithelial Transition

8.1 Introduction

The aim of this chapter was to investigate whether the pancreatic transcription factor *Isl-1* (islet-1) was expressed by the splenic mesenchyme within chimaeric recombinant organs. This gene is expressed in developing pancreatic islets (Karlsson, Thor et al. 1990; Ahlgren, Pfaff et al. 1997). The *Isl-1* gene encodes a transcription factor of the LIM homeodomain family, a class of proteins that control cell-fate decisions (Way and Chalfie 1988). Early expression is first seen in the pancreas of mouse embryos at E9, both in the dorsal pancreatic mesenchyme (DPM) and epithelium (DPE) (Ahlgren, Pfaff et al. 1997). Although *Isl-1* is not expressed in the ventral pancreatic mesenchyme (VPM), ventral pancreatic epithelial (VPE) *Isl-1* expression is first detected at E11, consistent with the one to two day delay in islet cell genesis in the ventral pancreatic bud (Herrera, Huarte et al. 1991; Ahlgren, Pfaff et al. 1997). Analysis of pancreatic epithelial endocrine cells (between E9.5-E13) shows *Isl-1* expression after their final mitotic division but before the onset of hormone-gene expression (Ahlgren, Pfaff et al. 1997). DPM expression is transient but is essential for its development and also for the generation of glucagon-expressing cells in the DPE. *Isl-1* expression in pancreatic epithelium is required for differentiation of islet cells, since pancreatic explants generated from *Isl-1*(-/-) mice are absent of insulin, glucagon and somatostatin expression (Ahlgren, Pfaff et al. 1997). Foregut *Isl-1* expression becomes more restricted during development, with expression in stomach mesenchyme, pancreatic mesenchyme and pancreatic epithelium at E11.5 in mice embryos, but then only in pancreatic epithelial precursors by E13.5 (Kim, Hebrok et al. 2000). *Isl-1* is also found in islet beta-cell progenitors and co-localises with insulin in differentiated beta-cells to maintain insulin production (Karlsson, Thor et al. 1990; Habener, Kemp et al. 2005). It is expressed in alpha,

beta, delta and pancreatic polypeptide cells of the adult islet (Dong, Asa et al. 1991; Thor, Ericson et al. 1991).

Previous study in this laboratory has shown up-regulation of *Isl-1* in stomach mesenchyme when recombined with pancreatic epithelia (Rowan-Hull, Rao et al. In Press). We were now interested to know whether this gene was also up-regulated in splenic mesenchyme, when recombined with pancreatic epithelia. This chapter addresses the hypothesis that *Isl-1 is up-regulated in the splenic mesenchyme of chimaeric recombinant organs*.

8.2 Methods

Recombinants were generated as detailed previously (2 quail spleens recombined with 2 chick pancreatic epithelia) and cultured for 7 days before fixation and cryo-sectioning (7-10μm). Immunocytochemistry for QCPN and *Isl-1* was performed, as detailed previously (Chapter 4, section 4.2.5). Slides were mounted in DAPI and analysed using fluorescence microscopy for antigen expression. Embryonic origins of splenic *Isl-1* positive cells were determined according to co-expression of nuclear *Isl-1* surrounding a QCPN nucleolus. Microdissected quail spleen controls were fixed either at day zero or after overnight culture. Controls underwent *Isl-1* immunocytochemistry and analysis as above.

8.3 *Isl-1* is up-regulated in splenic mesenchyme during iMET

3 / 9 (33%) spleen recombinants had co-localisation of QCPN and *Isl-1*, indicating that *Isl-1 is up-regulated in splenic mesenchyme when recombined with pancreatic epithelia* (see Table 8). All 9 recombinants had epithelial *Isl-1* expression, as expected from the pancreatic epithelia within the recombinant.

Interestingly, the *Isl-1* expression in the splenic mesenchyme did not appear to depend upon its proximity to epithelial pancreatic tissue in these experiments, since *Isl-1* positive cells of splenic origin were seen in their own separate clusters away from any neighbouring epithelia in 2 of 3 recombinants (see Figure 20B). The third recombinant showed *Isl-1* positive clusters of mixed origin but also isolated splenic mesenchymal cells showing *Isl-1* expression (see Figure 21).

In addition, a quantitative analysis of *Isl-1* expression was also performed. Within the three recombinants that demonstrated iMET, the mean number of all *Isl-1* +ve cells (regardless of origin) was 375 ± 150, and the mean number of splenic mesenchymal cells expressing *Isl-1* was 52 ± 38 (see Figure 19).

Six quail spleen controls were analysed for *Isl-1* expression (3 cultured overnight in normal RPMI, and 3 using RPMI with BIO; see Chapter 9: *A Preliminary Study of the Effect of a Wnt Agonist on islet Mesenchyme-to-Epithelial Transition in the Developing Spleen*) and were all negative, confirming clean separation.

Table 8 *Isl-1* expression in each splenic recombinant

Recombinant	QCPN -ve (epithelial) *Isl-1* +ve	"Mixed" cell clusters (*Isl-1* +ve cluster with some QCPN +ve contribution)	QCPN +ve (splenic) *Isl-1* +ve
1	+	-	+
2	+	-	+
3	+	-	-
4	+	-	-
5	+	-	-
6	+	-	-
7	+	-	-
8	+	+	+
9	+	-	-

Table 8 Cell clusters of QCPN –ve *Isl-1*+ve expression were seen in all recombinants, as expected from the pancreatic epithelium. However, cell clusters of QCPN+ve *Isl-1*+ve expression were seen in 3 / 9 recombinants, demonstrating up-regulation of this gene in the splenic mesenchyme.

Figure 19 Bar-chart showing number and origin of *Isl-1* positive cells for each recombinant

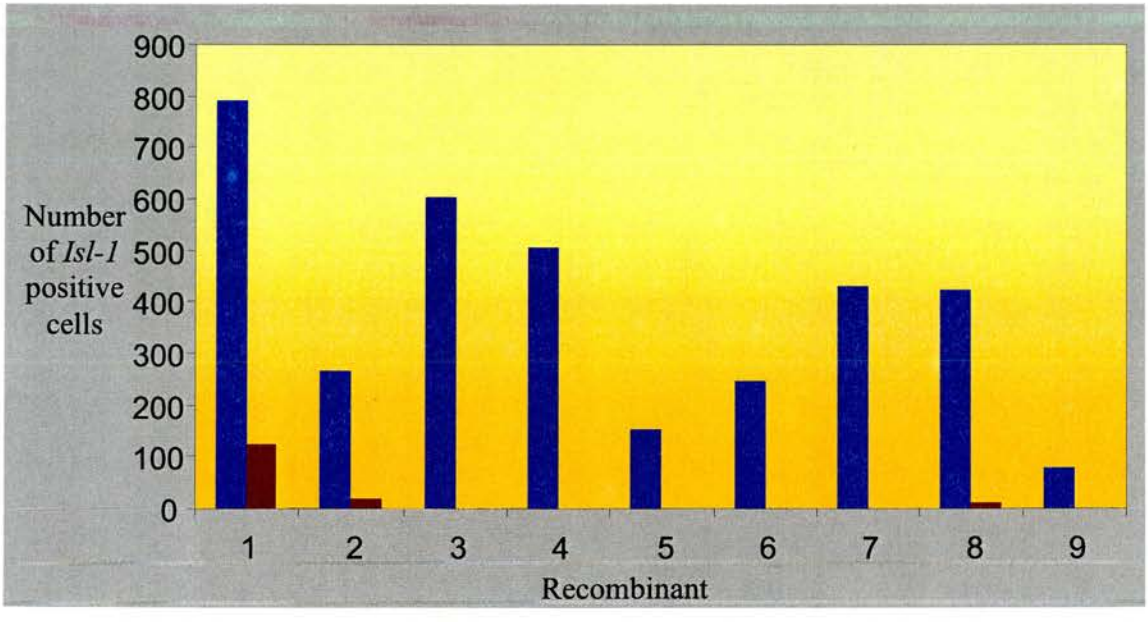


Fig 19 Bar-chart showing number of *Isl-1* positive cells of epithelial origin (in blue) and number of *Isl-1* positive cells of splenic origin (in red) for each recombinant

Figure 20 Expression pattern of *Isl-1*+ve cells of splenic origin

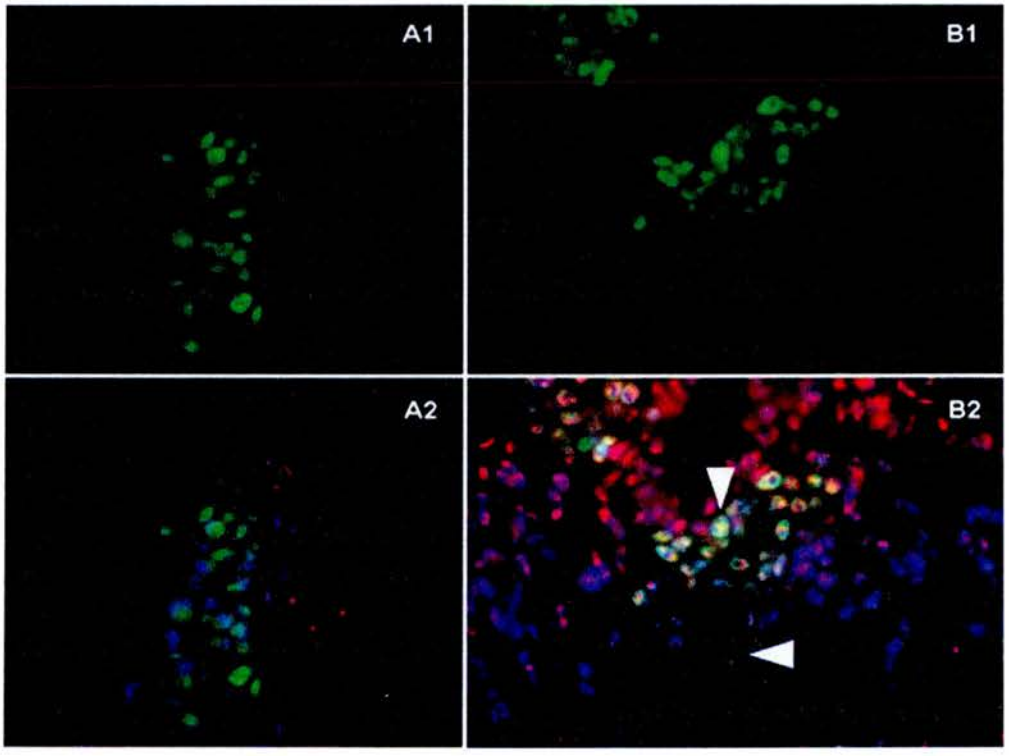


Fig 20 Splenic mesenchymal cells undergo iMET to express the pancreatic epithelial marker *Isl-1* in the absence of directly neighbouring pancreatic epithelial cells. B1: The splenic mesenchymal *Isl-1* +ve cells appeared in two recombinants in isolated clusters. B2: The same *Isl-1* clusters as B1, combined with red and blue channels. Examples of splenic cells expressing *Isl-1* are indicated by super-imposed white arrows. A1: An epithelial *Isl-1* cluster is shown for reference. A2: The same *Isl-1* cluster as A1, combined with red and blue channels (note the absence of QCPN positive nucleoli in the *Isl-1* positive cells). QCPN (red) identifies cells of splenic origin. DAPI (blue) labels the nucleus of all cells. Nuclear *Isl-1* expression is shown in green. Original magnification x63; section thickness 7-10μm.

Figure 21 Expression pattern of *Isl-1* +ve splenic mesenchymal cells in a recombinant

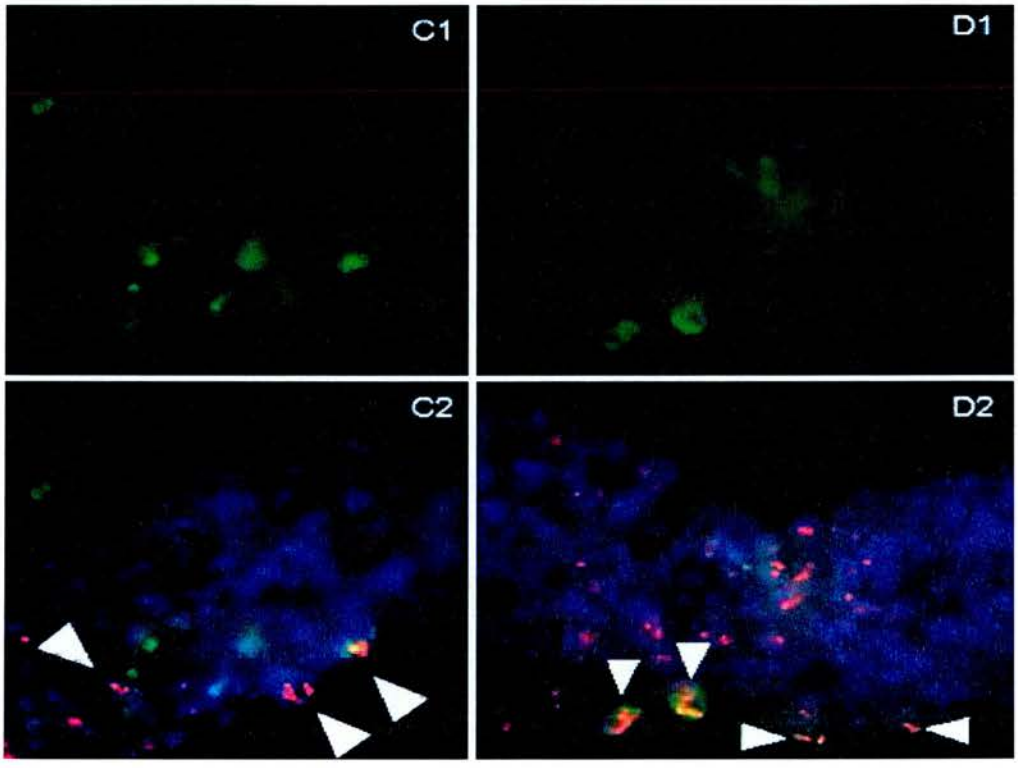


Fig 21 Splenic mesenchymal cells undergo iMET to express the pancreatic epithelial marker *Isl-1* in the absence of neighbouring pancreatic epithelial cells. C1: The mesenchymal *Isl-1* +ve cells in one recombinant appeared as isolated cells. C2: The same *Isl-1* +ve cells (indicated by super-imposed white arrows), with red and blue channels shown. D1: Another example of isolated mesenchymal *Isl-1* +ve cells. D2: The same *Isl-1* +ve cells (indicated by white arrows), with red and blue channels shown. QCPN (red) identifies cells of splenic origin. DAPI (blue) labels the nucleus of all cells. Nuclear *Isl-1* expression is shown in green. Original magnification x63; section thickness 7-10μm.

8.4 Discussion

Isl-1 was expressed in the splenic mesenchyme in a third of recombinants, confirming the hypothesis that *Isl-1 is up-regulated in the splenic mesenchyme when recombined with pancreatic epithelia*. This further demonstrates that the cells within splenic mesenchyme are able to adopt a pancreatic fate *in vitro* when recombined with pancreatic epithelium. These results also indicate that splenic mesenchyme can transdifferentiate into an endocrine epithelial fate, similar to the *Pdx-1* up-regulation findings in Chapter 7. Again, this presumably occurs under the influence of developmental signalling from the differentiating pancreatic epithelium. This might explain why *Isl-1* up-regulation was only seen in a third of cases, perhaps with some mesenchyme receiving more of these signals than others. A pancreas-specific epithelial signalling pathway could be confirmed by recombining splenic mesenchyme with non-pancreatic epithelium (stomach epithelium, for example) as a negative control.

In normal development, splenic mesenchyme does not express this homeobox gene (Ahlgren, Pfaff et al. 1997; Kim, Hebrok et al. 2000), and spleen controls in this experiment were also negative for *Isl-1*. The negative spleen controls also confirm clean separation and give additional confidence over the safety of dissection margins in this thesis.

Original studies of *Isl-1* gene expression suggested that it was restricted to cells of the endocrine pancreas (Karlsson, Thor et al. 1990). However, *Isl-1* expression is now known to be more widespread. Expression in the *adult* rat is not restricted to pancreatic islets, but has also been detected in extra-pancreatic locations, including

the nervous system (central and peripheral) and distal tubules of the kidney, but not in the spleen (Dong, Asa et al. 1991; Thor, Ericson et al. 1991). Mammalian *Isl-1* coding sequences, and their expression in non neuroendocrine lineages, are conserved between rat, hamster and human species (Wang and Drucker 1994). There are also reports of extra-pancreatic expression of *Isl-1* during development in stomach mesenchyme, forebrain, hypothalamus, pituitary and the pineal gland (Kim, Hebrok et al. 2000; Liu, Liu et al. 2005; Zhang, Liu et al. 2006; Elshatory and Gan 2008). Therefore, although *Isl-1* up-regulation in the splenic mesenchyme of our recombinants is most likely due to pancreatic islet reprogramming, an alternative fate cannot be entirely excluded; such as stomach mesenchyme, alternative endocrine (pituitary, pineal), neural (forebrain, hypothalamus) or inner ear. However, when interpreting the *Isl-1* expression in light of recombination with pancreatic epithelia, along with the findings of *Pdx-1* up-regulation and insulin expression in the splenic mesenchyme, the argument for pancreatic islet reprogramming is compelling.

Isl- 1 expression in foregut mesenchyme is poorly understood. Although the role of the mesenchyme in the regionalisation of both the gut endoderm / epithelium (Yasugi and Mizuno 2008) and the pancreas (Kumar, Jordan et al. 2003) have been examined, there is little evidence of the regulation of mesenchymal differentiation by the epithelium. However, it is interesting to note that patterning of the radial axis of the gut mesenchyme in the chick has been shown to be controlled by the epithelium (Fukuda, Tanigawa et al. 1998; Sukegawa, Narita et al. 2000).

Previous results from our laboratory demonstrate that stomach mesenchymal cells up-regulate *Isl-1* when recombined with pancreatic epithelium, but can also co-express

insulin (Rowan-Hull, Rao et al. In Press). It would be interesting to test this property in *Isl-1*+ve splenic mesenchyme cells. Our current understanding of iMET from experiments involving stomach mesenchyme / pancreatic epithelium recombinants is that signals from the pancreatic epithelium result in mesenchyme transition into an endocrine progenitor. In the splenic recombinants in this thesis, *Isl-1* was expressed both within large cellular clusters of splenic mesenchyme and also as isolated cells. This occurred in the absence of adjacent or neighbouring pancreatic epithelium, suggesting that splenic tissue may be able to transdifferentiate with greater independence from close epithelial-mesenchyme interactions. Theoretically, this may occur under the influence of soluble factors from the pancreatic epithelium, rather than direct cell-to-cell interactions. The observation of isolated cells expressing *Isl-1* may be explained by these cells receiving inadequate *FGF2* signals from the pancreatic epithelium, which are required for clustering to occur (Hardikar, Marcus-Samuels et al. 2003).

The ability of the splenic mesenchyme to transdifferentiate may be due to the very close relationship shared between the spleen and pancreas during normal development (see Chapter 2, section 2.5). Interestingly, previous study of stomach mesenchyme / pancreatic epithelia recombinants found separate *Barx-1* and *Isl-1* regions in the mesenchyme with no combined expression, indicating that *Barx-1* must be down-regulated in the mesenchyme before this same tissue undergoes *Isl-1* up-regulation (unpublished data, using *Barx-1 in situ* hybridisation, QCPN peroxidase reaction and *Isl-1* antibody immunohistochemistry). It would be interesting to investigate this same property in splenic mesenchyme / pancreatic epithelia recombinants, to see whether *Tlx-1* must be down-regulated before *Isl-1* is up-regulated. However, given the

dramatic and complete down-regulation of *Tlx-1* in the splenic mesenchyme (Chapter 6), it seems likely that a similar reprogramming sequence occurs, where the splenic identity is lost before the new pancreatic identity is achieved.

8.5 Conclusions

Isl-1 was expressed in the splenic mesenchyme in a third of recombinants, showing that *Isl-1 is up-regulated in the splenic mesenchyme when recombined with pancreatic epithelia.* Taken together with the previous *Pdx-1* up-regulation and insulin expression data, this result supports the finding that cells within splenic mesenchyme can be reprogrammed to adopt a pancreatic endocrine epithelial fate *in vitro* when recombined with pancreatic epithelium.

Chapter Nine

A preliminary study of the effect of a *Wnt* agonist on islet Mesenchyme-to-Epithelial Transition in the developing spleen

9.1 Introduction

The aim of this chapter was to begin a preliminary investigation into whether the addition of a *Wnt* (wingless-type) agonist to the culture medium would augment iMET. The hypothesis generated was that *iMET within chimaeric recombinant organs can be augmented by addition of a Wnt agonist to the culture medium.* This preliminary study analysed the number of splenic *Isl-1* expressing cells, in recombinants cultured with or without a *Wnt* agonist. Many studies have suggested that canonical *Wnt* signals play an important role in pancreas development. For example, a direct *Wnt* target, *Cdx4* (caudal type homeobox 4), acts to pattern the foregut endoderm (Kinkel, Eames et al. 2008). Furthermore, *Wnt* signalling is both necessary and sufficient for beta-cell proliferation (Rulifson, Karnik et al. 2007). Interestingly, *Wnt* signals also function in mesenchyme-to-epithelial transition in the developing metanephric kidney during nephron tubule formation (Kispert, Vainio et al. 1998).

Wnt molecules are a family of structurally-related secreted glycoproteins which are largely conserved in mammals (Piters, Boudin et al. 2008). They signal through binding Frizzled (*Fz*) receptor family members, which induces one of three known signalling pathways, including the canonical pathway studied here. This pathway depends on the translocation of a cytoplasmic complex, β-catenin, to the nucleus, where it binds lymphoid-enhancer binding factor / T-cell specific transcription factors (*Lef* / *Tcfs*), to control gene transcription (Eisenmann 2005). In the absence of *Wnt* signals, β-catenin is targeted for proteasomal degradation, through phosphorylation with the molecule Glycogen Synthase Kinase 3-β (GSK3β). The binding of a *Wnt*-ligand to an *Fz* receptor inactivates GSK3β, crippling the destruction complex, and

allowing β-catenin to translocate to the nucleus (see Figure 22). This study uses a GSK3β inhibitor, known as "Bio", as a *Wnt* agonist.

Figure 22 Flow-chart illustrating canonical *Wnt* signalling pathway

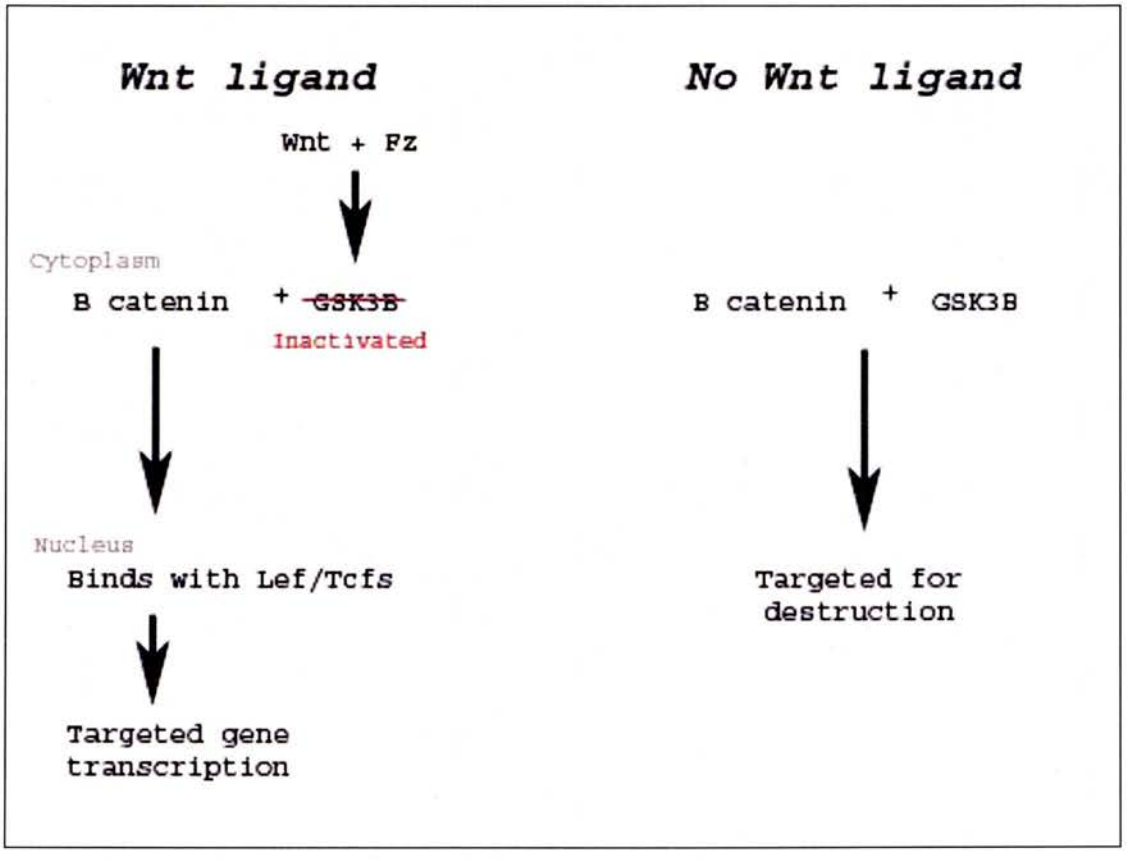


Fig 22 Flow-chart to illustrate canonical *Wnt* pathway. Absence of *Wnt* favours B-catenin destruction (via GSK3β). The presence of *Wnt* results in this molecule binding to *Fz* (*Frizzled*) receptor, inactivating GSK3β and hence preventing B-catenin destruction. B-catenin can then enter the nucleus, where it binds with *Lef / Tcfs* (lymphoid-enhancer binding factor / T-cell specific transcription factors) to control gene transcription pathways (Eisenmann 2005).

9.2 Methods

The materials and methods used in this section were identical to those used in Chapter 9, with the exception that Bio (Sigma, Dorset, UK), which is a GSK3β-inhibiting *Wnt* agonist, was added at a concentration of 1μM to the RPMI culture medium, where indicated. This included the RPMI used in the overnight agarose step and all RPMI used in the roller culture. A working concentration of 1μM was chosen following a study of the literature, which found working concentrations previously used in similar kinds of experiments ranging from 5nm to 2.5 μM (Heller, Klein et al. 2003; Naito, Shiojima et al. 2006; Slack, Lin et al. 2008; Ullmann, Gilles et al. 2008).

9.3 Results

Three splenic recombinants were successfully cultured in the presence of Bio. All three produced epithelial *Isl-1* +ve cell expression but only one recombinant (1/3, 33%) contained *Isl-1*+ve cells of a mesenchymal origin. Interestingly, this was only seen in one isolated cluster of approximately six mesenchymal *Isl-1* positive cells (see Figure 23).

Figure 23 Expression pattern of *Isl-1* positive splenic mesenchymal cells following culture with *Wnt* agonist

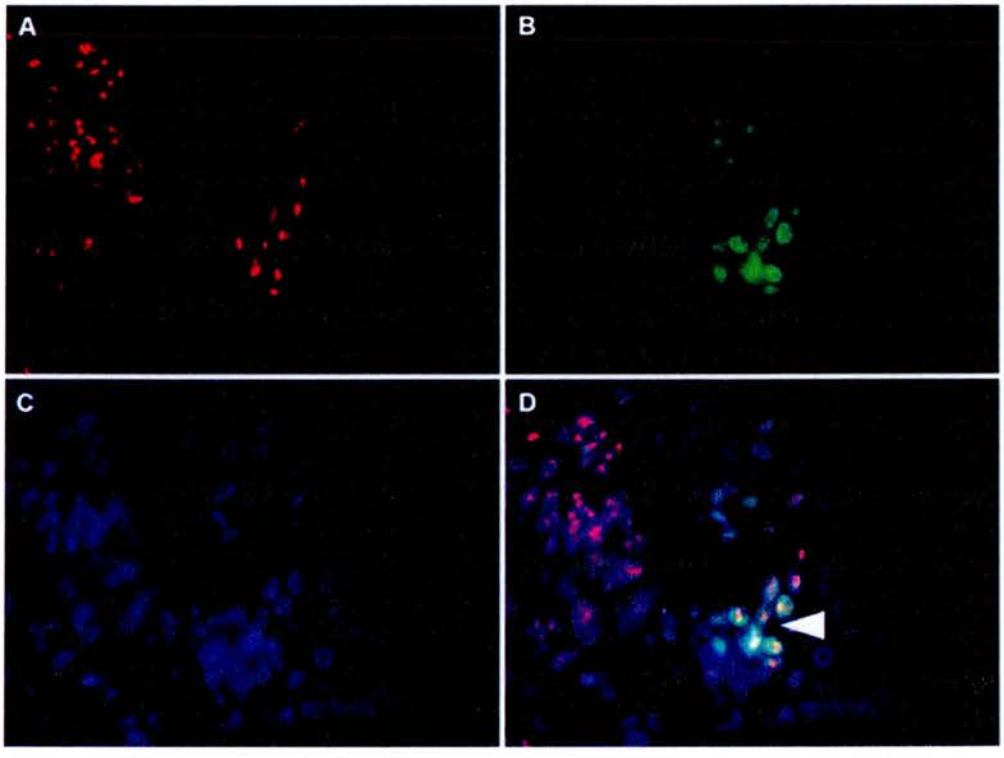


Fig 23 Expression pattern of *Isl-1*+ve splenic mesenchymal cells in a recombinant cultured in the presence of Bio. When recombined with chick pancreatic epithelium, quail splenic mesenchymal cells (A) undergo iMET to express the pancreatic epithelial marker *Isl-1* (B). This occurs in the absence of directly neighbouring pancreatic epithelial cells. The splenic mesenchymal cells also appear in isolated clusters (A) when Bio is added to the culture medium. A: Splenic quail QCPN in red, B: Nuclear *Isl-1* in green, C: Nuclear DAPI in blue, D: Combined image showing all three channels. Cluster of *Isl-1*+ve splenic mesenchymal cells indicated by super-imposed white arrow.

9.4 Discussion

The conclusions that can be drawn from this initial study of adding a *Wnt* agonist are limited, owing to the low number of recombinants. The experiment should be repeated during future work with a larger number of recombinants, before definite trends can be elucidated. Future work should also study the effect of adding a *Wnt* agonist on other pancreatic markers within the splenic mesenchyme of recombinants, such as insulin and *Pdx-1*. This study showed that 33% of recombinants contained *Isl-1*+ve cells of a splenic origin when cultured with Bio, the same percentage as that found without Bio (Chapter 8, section 8.3). Therefore, these preliminary results of three recombinant analysed for *Isl-1* suggest that *there may be no increase in Isl-1 expression observed in splenic mesenchyme when recombined with pancreatic epithelium in the presence of a Wnt agonist*, but further work is required.

Several studies implicate *Wnt* signals in pancreas development (Heiser, Lau et al. 2006; Jonckheere, Mayes et al. 2008). The addition of a *Wnt* agonist at this developmental stage may have been too late to produce a measurable effect using *Isl-1* in this experiment. For example, a recent study found that ectopic stabilisation of β-catenin *in vivo* within mouse pancreatic epithelium has divergent effects on organ growth and differentiation (Heiser, Lau et al. 2006). Early stabilisation of B-catenin resulted in a reduced pancreatic mass. Conversely, stabilisation later in pancreatic development resulted in enhanced proliferation and increased pancreatic mass. The apparent hinge point in that study occurred at mouse E12.5 (chick E3.75; just before the gestation that eggs are harvested in this thesis). Similar bi-phasic and stage-specific effects of *Wnt* signalling have been reported in the development of other organs, such as cardiomyogenesis and haematopoiesis (Naito, Shiojima et al. 2006).

Further characterisation of *Wnt* signalling during iMET would be useful, possibly through the addition of the *Wnt* agonist at differing time-points during iMET.

These preliminary results do not preclude the possibility that *Wnt* stimulation may be augmenting the expression of earlier pancreatic markers through an expansion of the endocrine progenitor pool. Analysis of other early pancreatic transcription factors, such as *Pdx-1* or *Ngn-3*, may help to clarify this.

If future tests are unable to demonstrate measurable differences in iMET, it may reflect either an inability of either or both the recombinant tissues to respond to *Wnt* signals in the culture medium, or perhaps a level of competition between these two tissues preventing a significant change in either one.

Since the pancreatic epithelium is known to be required for iMET to occur within the mesenchyme, it is reasonable to attribute *Wnt*-unresponsiveness to the epithelium. Some populations of fully-differentiated beta-cells that tolerate *in vivo* β-catenin stabilisation have been identified (Heiser, Lau et al. 2006). These fully differentiated cells display low nuclear and high cytoplasmic β-catenin levels, with no change in insulin expression. Some populations of fully differentiated beta-cells may express mechanisms which prevent stabilised β-catenin from entering the nucleus, rendering them unresponsive to increased canonical *Wnt* signals. Since Bio is a β-catenin stabiliser, this may also be the case in the experiments in this chapter.

Moreover, an eventual inability to demonstrate a significant change in iMET in response to *Wnt* may reflect a limitation of the experimental model. The pancreatic

mesenchyme that surrounds the developing pancreatic epithelium was replaced with splenic mesenchyme, which could render the pancreatic epithelium portion unresponsive to *Wnt* signals. Interestingly, a recent report has demonstrated that *mPygo2* (pygopus; an essential component of the nuclear complex necessary for canonical *Wnt* signalling) plays a role during the onset of endocrine development after mouse E12.5 (chick E3.75) (Jonckheere, Mayes et al. 2008). Pancreatic hypoplasia and decreased insulin expression in germ-line *mPygo2* mutants were reported and these defects were attributed to decreased proliferation of undifferentiated progenitors after mouse E13 (chick E4.75). However, the pancreas develops normally with epithelium-specific mutations, suggesting that *Wnt*-responsiveness in the endocrine pancreatic epithelium is an exclusive property of the surrounding pancreatic mesenchyme. The dependence of *mPygo2* upon *Wnt* signalling was confirmed, and canonical *Wnt* activity was demonstrated in the pancreatic mesenchyme when *mPygo2* mutant mice first show defects. The avian model in this thesis assesses endocrine differentiation at the equivalent developmental window. Therefore, separation of the pancreatic epithelium from its surrounding *Wnt*-responsive pancreatic mesenchyme during the recombinant chimaera construction may impair its ability to respond.

However, epithelial IPCs have already been observed in the microdissected pancreatic epithelium in this thesis at the time of pancreatic mesenchyme removal (see Chapter 4, Table 2). It is therefore likely that the pancreatic epithelial progenitors have already received the initial signals from the pancreatic mesenchyme at the time of dissection. In this case, the addition of our *Wnt* agonist may be too late to exert a noticeable effect in the absence of the pancreatic mesenchyme. Therefore, removal of the

pancreatic mesenchyme to create the recombinant tissue at this stage would not preclude initial *Wnt* signals being received from the pancreatic mesenchyme, but may preclude attempts to later augment them in the pancreatic epithelium.

9.5 Conclusions

The conclusions that can be drawn from this preliminary study of adding a *Wnt* agonist are limited, owing to the low number of surviving recombinants. The experiment therefore needs to be repeated during future work before definite trends can be elucidated. However, these preliminary results of three recombinants analysed for *Isl-1* suggest that *there may be no increase in Isl-1 expression observed in splenic mesenchyme when recombined with pancreatic epithelium in the presence of a Wnt agonist.*

SECTION FOUR

Conclusions and Future Research

Chapter Ten

Conclusions and Future Research

10.1 Summary of main results

The key findings in this thesis are summarised in Figure 24. Chapter 4 has outlined initial "proof of principle" experiments testing the first three experimental hypotheses (see Chapter 2, section 2.9, Table 1, hypotheses 1 to 3). The key findings show that the developing avian spleen is able to differentiate into insulin-producing cells (in almost a half of recombinants overall), and in an islet morphology, showing the first hypothesis to be true. The ratio of splenic mesenchyme to chick pancreatic epithelia does not appear to influence the percentage of recombinants in which iMET is observed (42% vs 33%), suggesting that signals from the differentiating pancreatic epithelium may be a key factor in this iMET process. However, doubling the amount of each tissue proportionately increases the percentage of recombinants in which this is observed (42% vs 80%). Although splenic iMET is observed in a proportionately higher percentage of recombinants when the amount of each tissue is increased, the actual percentage of IPC clusters in which iMET is observed significantly decreases when either the ratio of mesenchyme to epithelium, or the amount of tissue in each recombinant, was increased (37% vs 22% vs 13% respectively: see Chapter 4, Figure 12). Therefore, altering the ratio of splenic mesenchyme to chick pancreatic epithelium in the recombinants, or altering the overall amount of tissue in the recombinants, does alter the frequency of observed iMET, showing the second and third hypotheses to be true. These findings could be attributed to altered signalling between the two tissue types within the recombinants.

Section 3 outlines experiments investigating the molecular mechanisms behind splenic iMET, to test the final four experimental hypotheses (see Chapter 2, section 2.9, table 1, hypotheses 4 to 7). Experiments in Chapter 5 show how the *Tlx-1 in situ*

hybridisation reaction was optimised through modifications to the protocol. This involved the ubiquitous addition of EGTA to all fixative used, plus addition of glutaraldehyde to the fixative on Day One of the *in situ* protocol. Experiments in Chapter 5 then describe the characterisation of the splenic expression profile of *Tlx-1* during normal chick development during the developmental period in question (E4 to E11 inclusive). *Tlx-1* was found to be expressed in the spleen throughout this period, with no expression observed in other abdominal organs or surrounding tissues. Experiments in Chapter 6 show that the splenic mesenchyme is reprogrammed when recombined with pancreatic epithelia, through the dramatic down-regulation of *Tlx-1*, showing hypothesis 4 to be true. Experiments in Chapters 7 and 8 show the pancreatic endocrine transcription factors *Pdx-1* and *Isl-1* are up-regulated in the splenic mesenchyme when recombined with pancreatic epithelia, showing hypotheses 5 and 6 to be true. Finally, in Chapter 9, an attempt to augment iMET was made through the addition of a *Wnt* agonist to the culture medium. Preliminary data suggests that there may be no augmentation of *Isl-1* up-regulation. However, this experiment needs to be repeated as part of future work before hypothesis 7 can be accurately answered, along with a study of its effect on insulin-producing cells of splenic origin and *Pdx-1* regulation.

One potential weakness of the methodology used in this thesis is the risk of quail pancreatic contamination of the quail spleen during the microdissection steps of recombinant construction, as this might lead to false positives. However, no contamination was detected in any of the controls throughout the project, giving a very high level of confidence over the safety of the dissection margins (see Chapter 4, Table 3). Furthermore, a range of different pancreatic markers and a splenic marker

were used giving additional confidence, as well as great care being taken over the dissection, with a safety cuff of quail spleen (closest to the quail pancreas) being left behind during the dissection.

Taken together, these findings show that *splenic mesenchyme is able to differentiate into an insulin-producing pancreatic endocrine cell fate during development*, thus showing the principal hypothesis to be true.

Figure 24 Summary of key MD findings

1. The developing avian spleen is able to differentiate into insulin-producing cells and in an islet morphology
2. Altering the ratio of splenic mesenchyme to pancreatic epithelium in the recombinants alters the frequency of observed iMET
3. Altering the overall amount of tissue in the recombinants alters the frequency of observed iMET
4. The *Tlx-1 in situ* hybridisation reaction can be optimised through protocol modifications
5. *Tlx-1* is expressed in the spleen during normal chick development throughout the developmental period studied (day 4 to day 11 gestation) and in no other abdominal viscera
6. The splenic mesenchyme is reprogrammed when recombined with pancreatic epithelia, as shown by dramatic down-regulation of *Tlx-1*
7. *Pdx-1* is up-regulated in the splenic mesenchyme of chimaeric recombinant organs
8. *Isl-1* is up-regulated in the splenic mesenchyme of chimaeric recombinant organs
9. Further work is required to confirm the effect on iMET of adding a *Wnt* agonist to the culture medium (although preliminary data suggests that there may be no augmentation of *Isl-1* up-regulation)

10.2 Putative model of islet Mesenchyme-to-Epithelial Transition in the developing spleen

10.2.1 Islet mesenchyme-to-epithelial transition in the developing spleen : a new pathway for islet formation?

The finding that splenic mesenchyme is able to differentiate into an insulin-producing pancreatic endocrine cell fate during development is striking, as it offers a potential alternative pathway for islet differentiation through islet Mesenchyme-to-Epithelial Transition. It challenges the previously widely held dogma that islets are derived exclusively from endoderm (Percival and Slack 1999; Gu, Dubauskaite et al. 2002; Kawaguchi, Cooper et al. 2002). This finding also supports previous findings of iMET observed in both stomach and pancreatic mesenchyme in this model of pancreatic organogenesis (Lear, Jayanthi et al. 2004; Jayanthi, Rowan-Hull et al. 2005; Teague, Rowan-Hull et al. 2006). Islet MET from foregut mesenchyme may be a previously unrecognised pathway of normal islet development. Alternative pathways for islet neogenesis may exist in order to provide a survival advantage at times of stress or disease, for example. In addition, perhaps such pathways may also play a role in the "honeymoon" period observed during the onset of T1DM, where preservation of beta-cell function and reduced insulin requirements can initially be observed for a period after diagnosis (Heinze and Thon 1983; Abdul-Rasoul, Habib et al. 2006).

Until recently, islet differentiation was considered to be limited exclusively to differentiation of *Ngn3*-positive cells derived from *Pdx-1* expressing endoderm (Gu, Dubauskaite et al. 2002). However, a recent human genetic analysis has challenged

this dogma, finding beta-cell development in humans with homozygous mutations of the *Ngn-3* gene (Wang, Cortina et al. 2006). This is surprising, given that all endodermal islet precursors are *Ngn3*-positive (Gu, Dubauskaite et al. 2002). This finding may indicate a pathway for beta-cell formation that is independent of endodermal *Ngn3* precursors and iMET may be one such source. As already mentioned in Chapter 2, it is interesting to recall that *in vivo* ectopic islet differentiation within the spleen has previously been observed in nature. This has been observed naturally in some reptiles (Volk and Arquilla 1985) and also experimentally in mice lacking an exocrine pancreas (Krapp, Knofler et al. 1998). Together with the data in this thesis, these observations suggest that islet MET in the spleen may be able to occur naturally *in vivo*. Alternatively, the specific *in vitro* culture conditions and tissue manipulation within this model may alter the embryonic niche and provide signalling cues that allow islet MET to occur.

A possible alternative explanation to consider for our findings could be that splenic mesenchymal and pancreatic epithelial cells within the recombinants undergo spontaneous fusion. However, this is a very rare occurrence both in the chick-quail chimaera system (Grzeschik 1973) and in mesenchymal beta-cell differentiation (Ianus, Holz et al. 2003), and would not account for the frequency of splenic iMET observed in this thesis. Furthermore, it would seem unlikely that such a rare occurrence would also account for the accumulative body of evidence for iMET observed in other experiments (Kodama, Kuhtreiber et al. 2003; Lear, Jayanthi et al. 2004; Jayanthi, Rowan-Hull et al. 2005; Teague, Rowan-Hull et al. 2006).

The evidence supporting splenic iMET presented in this thesis may helpfully contribute to the debate over the role that adult mouse donor splenocytes may play in forming replacement islets to reverse diabetes in NOD mice (Kodama, Kuhtreiber et al. 2003; Chong, Shen et al. 2006; Couzin 2006; Faustman, Tran et al. 2006; Melton 2006; Nishio, Gaglia et al. 2006; Suri, Calderon et al. 2006). Although the evidence here is from an avian embryonic model, and not adult donor splenocytes, the ability of the developing avian embryonic spleen to be reprogrammed into an insulin-expressing pancreatic endocrine fate is noteworthy.

10.2.2 Hypothetical signalling pathways and molecular mechanisms behind islet Mesenchyme-to-Epithelial Transition in the developing spleen

The attempts in Section 3 to elucidate some of the molecular mechanisms behind this iMET process may also prove helpful in future islet neogenesis strategies using pluripotent cells. The presence of pancreatic epithelium was required for the splenic mesenchyme to be reprogrammed and undergo iMET and this reprogramming process presumably occurred under the influence of signalling by the differentiating pancreatic epithelium. However, it is important to remember that initial islet development has already occurred in the developing pancreatic tissue being harvested for recombinant construction (E4). Indeed, insulin expression was observed in the control chick pancreatic epithelia at the time of initial recombinant construction (see page 104 , Table 2). It is therefore unclear how the presence of the pancreatic epithelium within the recombinants could be influencing the splenic mesenchyme in my experiments, and which candidate signals from the differentiating pancreatic epithelium might be playing a role in the observed splenic iMET. Further work is

necessary to investigate this. However, expression of the very early pancreatic transcription factor *Pdx-1* is known to continue into adult life, and so it is feasible that early pancreatic factors such as these could continue to influence splenic mesenchyme within the recombinant during the culture period (Ohlsson, Karlsson et al. 1993).

The importance of differentiating pancreatic epithelium for MET is similar to a previous finding in this laboratory examining pancreatic mesenchyme (Jayanthi, Rowan-Hull et al. 2005). I postulate that these signals are responsible for the down-regulation of *Tlx-1* and up-regulation of *Pdx-1* and *Isl-1* in the splenic mesenchyme. The splenic mesenchyme appears very competent to be reprogrammed, as evidenced by the dramatic 100% down-regulation of *Tlx*-1 (Chapter 6). However, a range of pancreatic fate re-programming appears to occur, with *Pdx-1* being up-regulated in just over a half of recombinants (Chapter 7), *Isl-1* in a third of recombinants (Chapter 8) and differentiation into insulin-producing cells of splenic origin in approximately half of recombinants overall (Chapter 4), but in up to 80% of recombinants when two spleens are recombined with two pancreatic epithelia. This range of re-programming may be due to a range of pancreatic signalling being experienced by the splenic mesenchyme, from the pancreatic epithelia within the recombinants. As discussed, the role of increased *Wnt* signalling on splenic iMET is still unknown and requires further investigation. Therefore, a summarised hypothetical signalling pathway for splenic iMET is shown in Figure 25.

Figure 25 Hypothetical model of splenic iMET

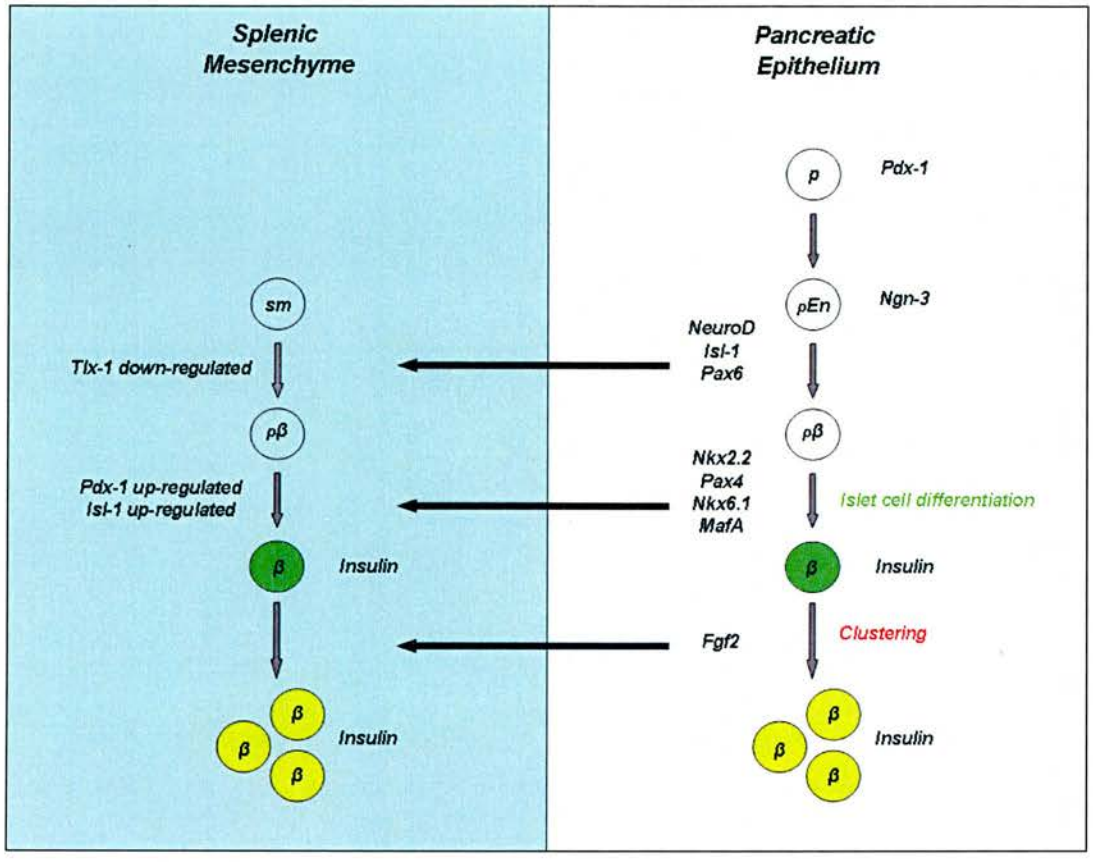


Fig 25 Hypothetical model of splenic iMET, based on the findings in this thesis. The splenic mesenchyme undergoes iMET under the influence of signals provided by the differentiating pancreatic epithelium; splenic mesenchyme in blue, pancreatic epithelium in white, pancreatic epithelial signalling on splenic mesenchyme shown in black arrows, sm = splenic mesenchyme, p = pancreatic precursor, pEn = pancreatic endocrine precursor, pβ = beta-cell precursor, β= beta-cell.

10.3 Potential future applications of this research in treating patients with Diabetes Mellitus

The data presented in this thesis indicates that the spleen may be a suitable tissue source from which replacement islets could potentially be derived. However, further work is needed to investigate splenic iMET, in particular further characterisation of the mechanisms controlling this (especially the required temporal pancreatic signalling cues) and whether the mature human spleen is capable of undergoing this iMET process (see section 10.4: *Future Research*). A desirable future islet phenotype derived from pluripotent cells would be safe and stable, capable of physiological insulin secretion kinetics, capable of maintaining euglycaemia and reversing Diabetes Mellitus.

A possible future treatment model could be envisaged, whereby a splenic biopsy is taken from the patient themselves shortly after diagnosis, either percutaneously (under radiological guidance) or surgically (via laparoscopy or laparotomy). This could be manipulated *in vitro* to produce the required islet phenotype and then transplanted back into the patient, via the portal vein for example (the route currently commonly utilised for allograft islet transplantation). Candidates for such a treatment would initially be T1DM patients, and T2DM patients requiring insulin. However, this treatment could eventually be made available to all patients with Diabetes Mellitus, if it provided euglycaemia and disease reversal, was low risk, and could be undertaken in a minimally-invasive manner.

If the new islets are susceptible to the same autoimmune attack as the native islets, they may require shielding from this (through concomitant immunosuppression, or perhaps via an immunomodulatory strategy or immunoisolation encapsulation) and "top-up" islet transfusions may even be required. Although islet regeneration offers the potential to reverse the disease, ameliorating the autoimmunity in T1DM will probably still be required to truly "cure" this disease.

In addition to autotranplantation, a splenic source of replacement islets could also be used for allotransplantation. Splenic tissue is readily available during multi-organ retrieval, but is generally not utilised. Therefore, this could be an abundant allograft tissue source in the future, if spleen-derived islet neogenesis is successfully realised.

10.4 Future research

Future work should involve *further characterisation of the splenic IPC clusters* produced via iMET. This would be useful to establish whether other islet cell types can also be produced and this could initially be undertaken using glucagon immunochemistry to detect alpha-cells (Teague, Rowan-Hull et al. 2007). In addition, it would be interesting to study whether these IPC clusters expressed the glucose transporter protein Glut2, which is a marker of mature beta-cells (Pang, Mukonoweshuro et al. 1994).

Further characterisation of the molecular mechanisms behind the splenic iMET observed in this thesis should be performed. Future research should initially further investigate the effect of adding a *Wnt* agonist to the culture medium on *Isl-1*

expression, but also on insulin and *Pdx-1* expression. Other extrapolation studies to augment or inhibit candidate signals should also be considered. The temporal pancreatic signalling cues need to be characterised. This might be achieved through using gene Micro-arrays or Real Time-Polymerase Chain Reaction techniques. Alternatively, differentiation protocols previously used to produce insulin-secreting islet-like clusters from human embryonic stem cells (Jiang, Au et al. 2007) could be adapted and applied to microdissected embryonic quail spleens.

Functional studies of the spleen-derived IPC clusters produced from splenic iMET should be performed to assess physiological islet hormone release (including insulin and glucagon). This could be performed through functional tests to measure insulin secretion upon glucose stimulation (using C-peptide enzyme-linked immunosorbent assay (ELISA), for example). The role of donor adult splenocytes to reverse diabetes in experimental models could also be re-addressed, following the conflicting results in NOD mice models (see Chapter 2, section 2.8).

Translational strategies should also be considered. This work has so far focussed on iMET in embryonic spleens, but future studies could investigate the adult spleen, initially using avian adult splenic tissue recombined with differentiating pancreatic epithelium. If adult splenocytes are unresponsive to such signalling cues, they may need to be dedifferentiated before becoming responsive to differentiating signals and this could be investigated in a similar approach to that used recently with human skin fibroblasts (Tateishi, He et al. 2008).

The findings in this thesis, together with the recent discovery that the mature human spleen continues to express *Tlx-1* throughout adulthood, may be a useful target for future bench-to-bedside translation strategies for this work (Dieguez-Acuna, Gygi et al. 2007; Lonyai, Kodama et al. 2008). The *Tlx-1* positive cell population in the mature human spleen could be manipulated *in vitro*, using an understanding of the molecular mechanisms behind splenic iMET outlined in this thesis, together with the future research detailed above. Together, these studies will help inform the design of novel future splenic iMET differentiation strategies to produce replacement islets for the treatment of T1DM.

Appendix I: Refereed publications

To date, the following refereed articles and abstracts have been published from the work presented in this thesis:

- Robertson SA, Rowan-Hull AM, Johnson PRV. **The spleen- a potential source of new islets for transplantation?** Journal Pediatric Surgery. 2008;43:274 -278.

- **Initial characterisation of the molecular mechanisms behind splenic islet mesenchyme-to-epithelial transition.** Stuart Robertson, Autumn Rowan-Hull, Paul Johnson. British Association of Paediatric Surgeons international meeting, Salamanca, Spain, 2nd-5th July 2008 (abstract)

Further publications are anticipated.

Appendix II: Presentations

Presentations given at scientific meetings from work presented in this thesis:

Oral presentations:

- **The lowly spleen - a potential source of new islets for transplantation?** Robertson SA, Rowan-Hull AM, Johnson PRV. Academic Paediatric Surgery Group national meeting. Alder-Hey Hospital, Liverpool, UK. Nov 9th 2006

- **The Spleen – a potential source of new islets for transplantation?** Robertson SA, Rowan-Hull AM, Johnson PRV. *Prize-winning oral presentation* in the Peter Paul Rickham prize competition at the British Association of Paediatric Surgeons international meeting, Edinburgh 17-20th July 2007

- **Islet mesenchyme-to-epithelial transition in the developing avian spleen** Oral presentation at the Academic Paediatric Surgeons Group national meeting, Oxford,17th January 2008

Poster presentations:

- **Initial characterisation of the molecular mechanisms behind splenic islet mesenchyme-to-epithelial transition.** Stuart Robertson, Autumn Rowan-Hull, Paul Johnson. British Association of Paediatric Surgeons international meeting, Salamanca, Spain, 2nd-5th July 2008

Appendix III: Supplemental solution information

Stock solutions

20x CMF-PBS (pH7.4) : 3M NaCl, 160mM Na_2P0_4, 340mM NaH_2P0_4 in molecular biology grade water (ddH_20); stored at room temperature (RT).

4% Phosphate-buffered paraformaldehyde (PFA) with EGTA: For 400mls: dissolve 16g PFA in 360mls ddH_20. Heat to dissolve with 5 drops 1M NaOH (takes approximately 20 minutes). Filter into measuring cylinder. Add 40mls 10x PBS. Add 1600 µl 0.5M EGTA (=2mM).

RNA probe synthesis solutions

Luria-Bertani (LB) medium: 10g bacto-tryptone, 5g yeast extract, 10g NaCl in 1L dH_20 in conical flask; autoclaved for 20 minutes, then stored at RT.

LB Agar: 10g bacto-tryptone, 5g yeast extract, 10g NaCl, 15g agar in 1L dH_20 with pH adjustment to 7.5 through additional NaOH titration; stored at RT and liquefied through microwave heating prior to use.

Plasmid DNA linearization reaction solution: added to eppendorf in following order; 6 µl ddH_20, 2 µl buffer, 10 µl DNA plasmid, 2 µl restriction enzyme. Enzymes and buffers stored at -20°C. Buffer chosen in accordance with NEBuffer chart (*http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/buffer_activity_restriction_enzymes.asp*). For example, NEBuffer chart recommended "Buffer 2" for *Tlx-1* plasmid linearization, with addition of 0.2 µl Bovine Serum Albumin.

RNA transcription reaction solution: added to eppendorf in following order; 9 µl ddH_20, 2 µl 10x transcription buffer; 2 µl digoxygenin (DIG), 5 µl linearised DNA, 1

µl RNase inhibitor, 1 µl of either T7 or T3 polymerase enzyme (depending on which linearised plasmid was being transcribed).

In situ hybridisation solutions and formulae

Prehybridisation solution (50mls): 25mls 50% formamide (Fluka), 12.5mls 5x SSC, 1g 2x Boehringer blocking powder, 50µl 0.1% Triton-X, 2.5mls 0.5% CHAPS (Sigma C3023) ,125µl 50µg / ml yeast RNA (sigma R6625), 500µl 5mM EDTA, 125µl 50µl/ml heparin; make up to 50 mls with ddH_20. Heated to 70°C to dissolve blocking powder.

2 x SSC + 0.1% CHAPS solution (50mls): 5mls 20x SSC, 0.5mls 10% CHAPS; made up to 50 mls with ddH_20.

0.2 x SSC + 0.1% CHAPS solution (50mls): 0.5mls 20x SSC, 0.5mls 10% CHAPS; made up to 50mls with ddH_20.

TBTx solution: 50mM Tris 7.4, 0.1% TritonX-100, 150mM NaCl; required volume of ddH_20 added.

Pre-absorption of antibody solution (10mls): 1ml 10% sheep serum, 0.2g 2% Bovine Serum Albumin, 9mls TBTx, 15mg Embryo powder, 5µl Anti-DIG Fab fragment. Rocked at 4°C for 3 hours. Centrifuged for 10 minutes to remove embryo powder debris at end.

Embryo powder (should match species eg: chick / quail): Embryos homogenised ranging from day 4 to day 6 gestation (half chick, half quail) in minimal volume PBS (RNase free). 4x volume of ice-cold acetone added. Mixed and incubated on ice for 30 minutes. Centrifuged at 10,000g for 10 minutes (in eppendorfs). Supernatant removed and discarded. Pellet washed with ice-cold acetone and centrifuged at 8,000g

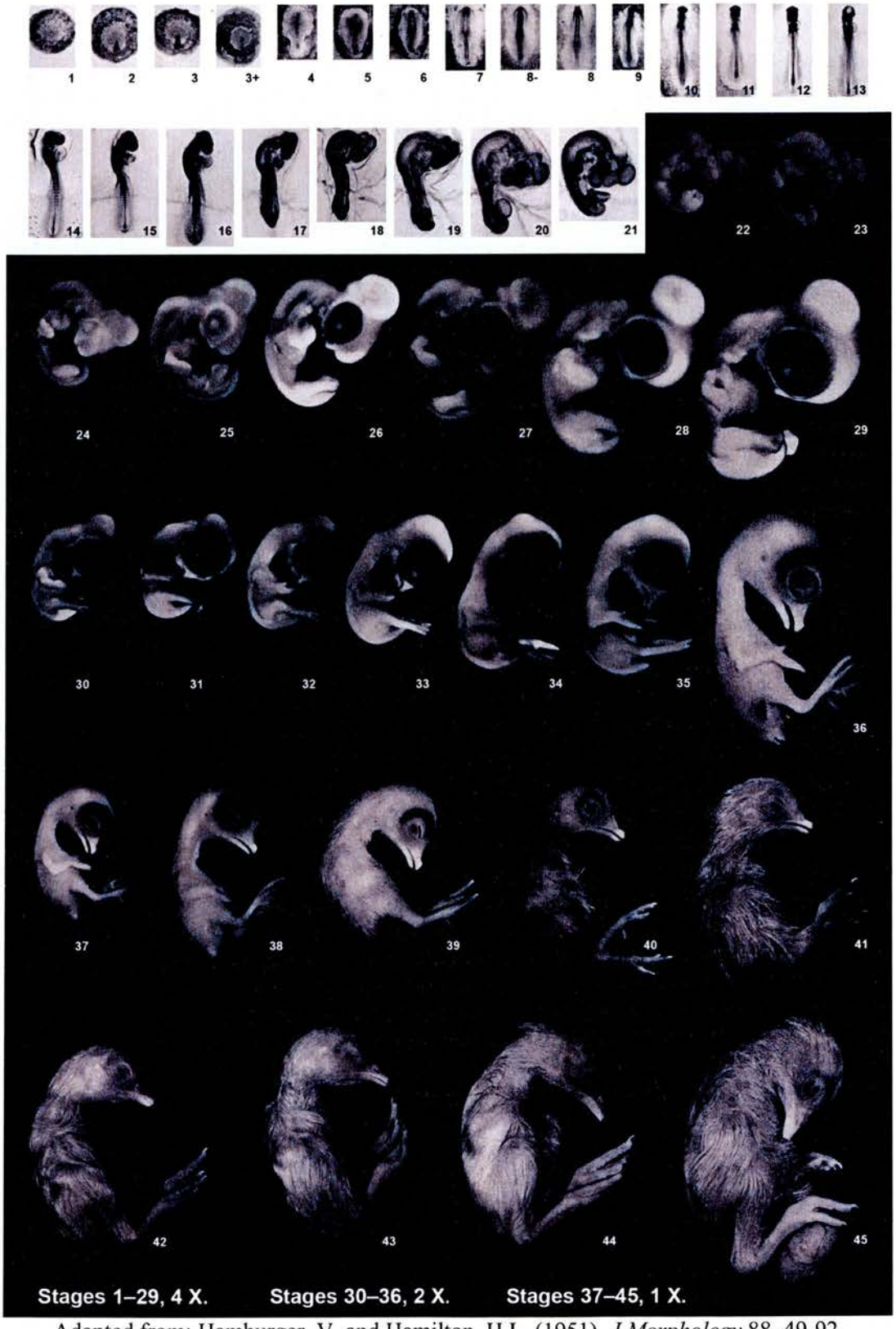
for 10 minutes. Pellet spread out and ground into fine powder on filter paper. Allowed to air dry. Stored in air-tight tube at 4°C.

Solution 1 (50mls): 25mls Formamide, 12.5mls 20x SSC, 50µl TritonX-100, 2.5mls 10% CHAPS; make up to 50 mls with ddH_20.

AP buffer (50mls): 5mls 1M Tris pH 9.5, 1ml 5M NaCl, 500µl $Tween_{20}$, 1.25mls 2M MgCl; make up to 50 mls with ddH_20.

Colour reaction solution (1ml): 1ml AP buffer, 3.5µl BCIP (100% DMF 50mg/ml), 4.5µl NBT (70% DMF 75mg/ml).

Appendix IV: Hamburger Hamilton chick staging chart



Adapted from: Hamburger, V. and Hamilton, H.L. (1951). *J Morphology* 88, 49-92.
(Courtesy of WJ Teague)

Acknowledgements

I would particularly like to thank the following people for their help and support during this research:

Paul Johnson, my MD supervisor in Oxford, for his supervision, mentorship, advice, support and friendship.

Robert Minns, for being my MD supervisor in Edinburgh.

Autumn Rowan-Hull, for friendship, support, guidance, supervision and tutorage in developmental biology laboratory skills.

Kingsley Micklem, for help with imaging and the preparation and publication of image data.

Paul D'Alessandro, an MSc student in the laboratory, for his helpful contribution to the immunohistochemistry and analysis during the *Isl-1* and *Wnt* experiments.

Juris Galvanovskis, for help with the statistical analysis in Chapter 4.

Manuela Carvalho-Gaspar, for her assistance with the *Tlx-1* plasmid sequence alignment.

Derek Gray, NDS Professor of Experimental Surgery, for giving generously of his time to proof-read the thesis and for his helpful comments.

Steve Hughes and *Sarah Cross,* for making islet isolations cheerful and for their useful contributions at laboratory meetings.

My parents, *Iain and Judith Robertson*, for their love and prayerful support.

Emma Simmons, for her love and support during the long hours of thesis writing.

References

Abdul-Rasoul, M., H. Habib, et al. (2006). "'The honeymoon phase' in children with type 1 diabetes mellitus: frequency, duration, and influential factors." Pediatr Diabetes **7**(2): 101-7.

Abraham, E. J., S. Kodama, et al. (2004). "Human pancreatic islet-derived progenitor cell engraftment in immunocompetent mice." Am J Pathol **164**(3): 817-30.

Ackermann Misfeldt, A., R. H. Costa, et al. (2008). "{beta}-Cell Proliferation, but not Neogenesis, Following 60% Partial Pancreatectomy is Impaired in the Absence of FoxM1." Diabetes.

Adams, A. B., N. Shirasugi, et al. (2002). "Calcineurin inhibitor-free CD28 blockade-based protocol protects allogeneic islets in nonhuman primates." Diabetes **51**(2): 265-70.

Afelik, S., Y. Chen, et al. (2006). "Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue." Genes Dev **20**(11): 1441-6.

Affolter, M., S. Bellusci, et al. (2003). "Tube or not tube: remodeling epithelial tissues by branching morphogenesis." Dev Cell **4**(1): 11-8.

Ahlgren, U., J. Jonsson, et al. (1996). "The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice." Development **122**(5): 1409-16.

Ahlgren, U., J. Jonsson, et al. (1998). "beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes." Genes Dev **12**(12): 1763-8.

Ahlgren, U., S. L. Pfaff, et al. (1997). "Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells." Nature **385**(6613): 257-60.

Ai, C., I. Todorov, et al. (2007). "Human marrow-derived mesodermal progenitor cells generate insulin-secreting islet-like clusters in vivo." Stem Cells Dev **16**(5): 757-70.

Akazawa, H., I. Komuro, et al. (2000). "Targeted disruption of the homeobox transcription factor Bapx1 results in lethal skeletal dysplasia with asplenia and gastroduodenal malformation." Genes Cells **5**(6): 499-513.

Amit, M., M. K. Carpenter, et al. (2000). "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture." Dev Biol **227**(2): 271-8.

Andrew, A. (1975). "APUD cells in the endocrine pancreas and the intestine of chick embryos." Gen Comp Endocrinol **26**(4): 485-95.

Andrew, A., B. B. Rawdon, et al. (1994). "Failure of insulin cells to develop in cultured embryonic chick pancreas: a model system for the detection of factors supporting insulin cell differentiation." In Vitro Cell Dev Biol Anim **30A**(10): 664-70.

Ang, S. L. and J. Rossant (1994). "HNF-3 beta is essential for node and notochord formation in mouse development." Cell **78**(4): 561-74.

Ang, S. L., A. Wierda, et al. (1993). "The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins." Development **119**(4): 1301-15.

Aoi, T., K. Yae, et al. (2008). "Generation of Pluripotent Stem Cells from Adult Mouse Liver and Stomach Cells." Science.

Apelqvist, A., U. Ahlgren, et al. (1997). "Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas." Curr Biol **7**(10): 801-4.

Apelqvist, A., H. Li, et al. (1999). "Notch signalling controls pancreatic cell differentiation." Nature **400**(6747): 877-81.

Armstrong, J. F., K. Pritchard-Jones, et al. (1993). "The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo." Mech Dev **40**(1-2): 85-97.

Asayesh, A., J. Sharpe, et al. (2006). "Spleen versus pancreas: strict control of organ interrelationship revealed by analyses of Bapx1-/- mice." Genes Dev **20**(16): 2208-13.

Aspinwall, C. A., J. R. Lakey, et al. (1999). "Insulin-stimulated insulin secretion in single pancreatic beta cells." J Biol Chem **274**(10): 6360-5.

Assady, S., G. Maor, et al. (2001). "Insulin production by human embryonic stem cells." Diabetes **50**(8): 1691-7.

Atkinson, M. A. and G. S. Eisenbarth (2001). "Type 1 diabetes: new perspectives on disease pathogenesis and treatment." Lancet **358**(9277): 221-9.

Atkinson, M. A. and N. K. Maclaren (1994). "The pathogenesis of insulin-dependent diabetes mellitus." N Engl J Med **331**(21): 1428-36.

Atouf, F., C. H. Park, et al. (2007). "No evidence for mouse pancreatic beta-cell epithelial-mesenchymal transition in vitro." Diabetes **56**(3): 699-702.

Aylsworth, A. S. (2001). "Clinical aspects of defects in the determination of laterality." Am J Med Genet **101**(4): 345-55.

Badet, L., T. T. Titus, et al. (2001). "Transplantation of mouse pancreatic islets into primates--in vivo and in vitro evaluation." Transplantation **72**(12): 1867-74.

Baeyens, L., S. De Breuck, et al. (2005). "In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells." Diabetologia **48**(1): 49-57.

Bai, L., K. Maedler, et al. (2004). "Expression of Fas but not Fas ligand on fetal pig beta cells." Xenotransplantation **11**(5): 426-35.

Ball, E. M. and G. P. Risbridger (2001). "Activins as regulators of branching morphogenesis." Dev Biol **238**(1): 1-12.

Bannerman, R. M., G. Keusch, et al. (1967). "Thalassemia intermedia, with iron overload, cardiac failure, diabetes mellitus, hypopituitarism and porphyrinuria." Am J Med **42**(3): 476-86.

Banting, F. G. and C. H. Best (1922). "The internal secretion of the pancreas." J Lab Clin Med **7**: 251-266.

Banting, F. G., C. H. Best, et al. (1922). "Pancreatic extracts in the treatment of diabetes mellitus: preliminary report " CMAJ **121**: 141-146.

Barth, J. and R. Ivarie (1994). "Polyvinyl alcohol enhances detection of low abundance transcripts in early stage quail embryos in a nonradioactive whole mount in situ hybridization technique." Biotechniques **17**(2): 324, 326-7.

Battula, V. L., P. M. Bareiss, et al. (2007). "Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation." Differentiation **75**(4): 279-91.

Beattie, G. M., A. D. Lopez, et al. (1999). "Transplantation of human fetal pancreas: fresh vs. cultured fetal islets or ICCS." J Mol Med **77**(1): 70-3.

Beauchemin, M., N. Noiseux, et al. (1994). "Expression of Hox A11 in the limb and the regeneration blastema of adult newt." Int J Dev Biol **38**(4): 641-9.

Beaupain, D. and F. Dieterlen-Lievre (1974). "[An immunocytological study of differentiation of the endocrine pancreas of the chick embryo. II. Glucagon]." Gen Comp Endocrinol **23**(4): 421-31.

Bell, E. T. (1942). "Renal lesions in diabetes mellitus." Am J Pathol **18**: 744-745.

Bennet, W., A. Bjorkland, et al. (2000). "A comparison of fetal and adult porcine islets with regard to Gal alpha (1,3)Gal expression and the role of human immunoglobulins and complement in islet cell cytotoxicity." Transplantation **69**(8): 1711-7.

Berthoud, H. R., E. R. Trimble, et al. (1980). "Cephalic-phase insulin secretion in normal and pancreatic islet-transplanted rats." Am J Physiol **238**(4): E336-40.

Bhushan, A., N. Itoh, et al. (2001). "Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis." Development **128**(24): 5109-17.

Bonner-Weir, S., A. Inada, et al. (2008). "Transdifferentiation of pancreatic ductal cells to endocrine beta-cells." Biochem Soc Trans **36**(Pt 3): 353-6.

Bonner-Weir, S. and A. Sharma (2006). "Are there pancreatic progenitor cells from which new islets form after birth?" Nat Clin Pract Endocrinol Metab **2**(5): 240-1.

Bonner-Weir, S., M. Taneja, et al. (2000). "In vitro cultivation of human islets from expanded ductal tissue." Proc Natl Acad Sci U S A **97**(14): 7999-8004.

Bonner-Weir, S., E. Toschi, et al. (2004). "The pancreatic ductal epithelium serves as a potential pool of progenitor cells." Pediatr Diabetes **5 Suppl 2**: 16-22.

Bonner-Weir, S. and G. C. Weir (2005). "New sources of pancreatic beta-cells." Nat Biotechnol **23**(7): 857-61.

Boorman, C. J. and S. M. Shimeld (2002). "The evolution of left-right asymmetry in chordates." Bioessays **24**(11): 1004-11.

Bouwens, L. and D. G. Pipeleers (1998). "Extra-insular beta cells associated with ductules are frequent in adult human pancreas." Diabetologia **41**(6): 629-33.

Bouwens, L. and I. Rooman (2005). "Regulation of pancreatic beta-cell mass." Physiol Rev **85**(4): 1255-70.

Brazelton, T. R. and H. M. Blau (2005). "Optimizing techniques for tracking transplanted stem cells in vivo." Stem Cells **23**(9): 1251-65.

Brendolan, A., E. Ferretti, et al. (2005). "A Pbx1-dependent genetic and transcriptional network regulates spleen ontogeny." Development **132**(13): 3113-26.

Brendolan, A., M. M. Rosado, et al. (2007). "Development and function of the mammalian spleen." Bioessays **29**(2): 166-77.

Brinkmann, V., H. Foroutan, et al. (1995). "Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells." J Cell Biol **131**(6 Pt 1): 1573-86.

Brolen, G. K., N. Heins, et al. (2005). "Signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing beta-cell-like cells." Diabetes **54**(10): 2867-74.

Brooks-Worrell, B. M., K. P. Peterson, et al. (2000). "Reactivation of type 1 diabetes in patients receiving human fetal pancreatic tissue transplants without immunosuppression." Transplantation **69**(9): 1824-9.

Brown, R. S., J. Edwards, et al. (2002). "Routine acid decalcification of bone marrow samples can preserve DNA for FISH and CGH studies in metastatic prostate cancer." J Histochem Cytochem **50**(1): 113-5.

Brown, W. R., S. J. Hubbard, et al. (2003). "The chicken as a model for large-scale analysis of vertebrate gene function." Nat Rev Genet **4**(2): 87-98.

Bru, T., C. Clarke, et al. (2008). "Rapid induction of pluripotency genes after exposure of human somatic cells to mouse ES cell extracts." Exp Cell Res.

Brunicardi, F. C., R. M. Kleinman, et al. (1994). "The inhibitory role of intraislet somatostatin on glucagon secretion in the isolated perfused human pancreas." Transplant Proc **26**(6): 3451-2.

Burns, C. J., S. J. Persaud, et al. (2004). "Stem cell therapy for diabetes: do we need to make beta cells?" J Endocrinol **183**(3): 437-43.

Burra, P., A. Buda, et al. (2006). "Occurrence of post-transplant lymphoproliferative disorders among over thousand adult recipients: any role for hepatitis C infection?" Eur J Gastroenterol Hepatol **18**(10): 1065-70.

Butler, A. E., J. Janson, et al. (2003). "Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid." Diabetes **52**(9): 2304-14.

Calafiore, R., G. Basta, et al. (2006). "Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: first two cases." Diabetes Care **29**(1): 137-8.

Calne, R., S. D. Moffatt, et al. (2000). "Prope tolerance with induction using Campath 1H and low-dose cyclosporin monotherapy in 31 cadaveric renal allograft recipients." Nippon Geka Gakkai Zasshi **101**(3): 301-6.

Chadburn, A. (2000). "The spleen: anatomy and anatomical function." Semin Hematol **37**(1 Suppl 1): 13-21.

Chao, C. S., Z. L. Loomis, et al. (2007). "Genetic identification of a novel NeuroD1 function in the early differentiation of islet alpha, PP and epsilon cells." Dev Biol **312**(2): 523-32.

Chase, L. G., F. Ulloa-Montoya, et al. (2007). "Islet-derived fibroblast-like cells are not derived via epithelial-mesenchymal transition from Pdx-1 or insulin-positive cells." Diabetes **56**(1): 3-7.

Cheung, A. T., B. Dayanandan, et al. (2000). "Glucose-dependent insulin release from genetically engineered K cells." Science **290**(5498): 1959-62.

Chiang, M. K. and D. A. Melton (2003). "Single-cell transcript analysis of pancreas development." Dev Cell **4**(3): 383-93.

Chong, A. S., J. Shen, et al. (2006). "Reversal of diabetes in non-obese diabetic mice without spleen cell-derived beta cell regeneration." Science **311**(5768): 1774-5.

Chung, Y., I. Klimanskaya, et al. (2008). "Human embryonic stem cell lines generated without embryo destruction." Cell Stem Cell **2**(2): 113-7.

Chung, Y., I. Klimanskaya, et al. (2006). "Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres." Nature **439**(7073): 216-9.

CITR (2008). "Collaborative Islet Transplant Registry 2008 report."

Civardi, G., D. Vallisa, et al. (2001). "Ultrasound-guided fine needle biopsy of the spleen: high clinical efficacy and low risk in a multicenter Italian study." Am J Hematol **67**(2): 93-9.

Clark, A., C. A. Wells, et al. (1988). "Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes." Diabetes Res **9**(4): 151-9.

Close, N., R. Alejandro, et al. (2007). "Second annual analysis of the collaborative islet transplant registry." Transplant Proc **39**(1): 179-82.

Cockell, M., D. Stolarczyk, et al. (1995). "Binding sites for hepatocyte nuclear factor 3 beta or 3 gamma and pancreas transcription factor 1 are required for efficient expression of the gene encoding pancreatic alpha-amylase." Mol Cell Biol **15**(4): 1933-41.

Collombat, P., A. Mansouri, et al. (2003). "Opposing actions of Arx and Pax4 in endocrine pancreas development." Genes Dev **17**(20): 2591-603.

Copland, P. S. (2004). "The Roman Catholic Church and embryonic stem cells." J Med Ethics **30**(6): 607-8.

Cornelius, J. G., V. Tchernev, et al. (1997). "In vitro-generation of islets in long-term cultures of pluripotent stem cells from adult mouse pancreas." Horm Metab Res **29**(6): 271-7.

Coulombe, M. and R. G. Gill (2004). "The immunobiology of pancreatic islet transplantation." Advances in Experimental Medicine & Biology **552**: 154-69.

Couzin, J. (2006). "Immunology. Diabetes studies conflict on power of spleen cells." Science **311**(5768): 1694.

Crisera, C. A., A. S. Kadison, et al. (2000). "Expression and role of laminin-1 in mouse pancreatic organogenesis." Diabetes **49**(6): 936-44.

D'Amour, K. A., A. D. Agulnick, et al. (2005). "Efficient differentiation of human embryonic stem cells to definitive endoderm." Nat Biotechnol **23**(12): 1534-41.

Davies, J. A. and C. E. Fisher (2002). "Genes and proteins in renal development." Exp Nephrol **10**(2): 102-13.

Davies, J. A., A. D. Perera, et al. (1999). "Mechanisms of epithelial development and neoplasia in the metanephric kidney." Int J Dev Biol **43**(5): 473-8.

DCCTRG (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. Diabetes Control and Complications Trial Research Group. N Engl J Med. **329:** 977-86.

DCCTRG (1997). "Hypoglycemia in the Diabetes Control and Complications Trial. The Diabetes Control and Complications Trial Research Group." Diabetes **46**(2): 271-86.

De Paepe, M. E., B. Keymeulen, et al. (1995). "Proliferation and hypertrophy of liver cells surrounding islet grafts in diabetic recipient rats." Hepatology **21**(4): 1144-53.

Dear, T. N., W. H. Colledge, et al. (1995). "The Hox11 gene is essential for cell survival during spleen development." Development **121**(9): 2909-15.

Delis, S., G. W. Burke, 3rd, et al. (2006). "Bone marrow-induced tolerance in the era of pancreas and islets transplantation." Pancreas **32**(1): 1-8.

Denholm, B., V. Sudarsan, et al. (2003). "Dual origin of the renal tubules in Drosophila: mesodermal cells integrate and polarize to establish secretory function." Curr Biol **13**(12): 1052-7.

Derubeis, A. R., M. Mastrogiacomo, et al. (2003). "Osteogenic potential of rat spleen stromal cells." Eur J Cell Biol **82**(4): 175-81.

Dieguez-Acuna, F. J., S. P. Gygi, et al. (2007). "Splenectomy: a new treatment option for ALL tumors expressing Hox-11 and a means to test the stem cell hypothesis of cancer in humans." Leukemia **21**(10): 2192-4.

Diesen, D. L., S. A. Zimmerman, et al. (2008). "Partial splenectomy for children with congenital hemolytic anemia and massive splenomegaly." J Pediatr Surg **43**(3): 466-72.

Dieterlen-Lievre, F. and D. Beaupain (1974). "[Immunocytological study of the differentiation of the endocrine pancreas in the chick embryo. I. Islands of Langerhans]." Gen Comp Endocrinol **22**(1): 62-9.

Djordjevic, P. B., N. M. Lalic, et al. (2004). "Human fetal islet transplantation in type 1 diabetic patients: comparison of metabolic effects between single and multiple implantation regimens." Transplant Proc **36**(9): 2869-73.

Dombrowski, F. (2000). "[Tumorigenesis after intra-portal pancreatic islet transplantation in diabetic rats]." Verh Dtsch Ges Pathol **84**: 136-50.

Donath, M. Y. and P. A. Halban (2004). "Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications." Diabetologia **47**(3): 581-9.

Dong, J., S. L. Asa, et al. (1991). "Islet cell and extrapancreatic expression of the LIM domain homeobox gene isl-1." Mol Endocrinol **5**(11): 1633-41.

Dor, Y. (2006). "beta-Cell proliferation is the major source of new pancreatic beta cells." Nat Clin Pract Endocrinol Metab **2**(5): 242-3.

Dor, Y., J. Brown, et al. (2004). "Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation." Nature **429**(6987): 41-6.

Dor, Y. and D. A. Melton (2004). "How important are adult stem cells for tissue maintenance?" Cell Cycle **3**(9): 1104-6.

Dordevic, P. B., N. M. Lalic, et al. (1995). "Human fetal islet transplantation in IDDM patients: an 8-year experience." Transplant Proc **27**(6): 3146-7.

Draper, J. S., K. Smith, et al. (2004). "Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells." Nat Biotechnol **22**(1): 53-4.

Drukker, M. (2004). "Immunogenicity of human embryonic stem cells: can we achieve tolerance?" Springer Semin Immunopathol **26**(1-2): 201-13.

Drukker, M. and N. Benvenisty (2004). "The immunogenicity of human embryonic stem-derived cells." Trends Biotechnol **22**(3): 136-41.

Drukker, M., H. Katchman, et al. (2006). "Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells." Stem Cells **24**(2): 221-9.

Drukker, M., G. Katz, et al. (2002). "Characterization of the expression of MHC proteins in human embryonic stem cells." Proc Natl Acad Sci U S A **99**(15): 9864-9.

Dube, I. D., S. Kamel-Reid, et al. (1991). "A novel human homeobox gene lies at the chromosome 10 breakpoint in lymphoid neoplasias with chromosomal translocation t(10;14)." Blood **78**(11): 2996-3003.

Dufrane, D., R. M. Goebbels, et al. (2006). "Six-month survival of microencapsulated pig islets and alginate biocompatibility in primates: proof of concept." Transplantation **81**(9): 1345-53.

Dupin, E., C. Ziller, et al. (1998). "The avian embryo as a model in developmental studies: chimeras and in vitro clonal analysis." Curr Top Dev Biol **36**: 1-35.

Eberhardt, M., P. Salmon, et al. (2006). "Multipotential nestin and Isl-1 positive mesenchymal stem cells isolated from human pancreatic islets." Biochem Biophys Res Commun **345**(3): 1167-76.

EDICSG (2002). "Effect of intensive therapy on the microvascular complications of type 1 diabetes mellitus (Epidemiology of Diabetes Interventions and Complications Study Group)." Jama **287**(19): 2563-9.

EDICSG (2003). "Sustained effect of intensive treatment of type 1 diabetes mellitus on development and progression of diabetic nephropathy: the Epidemiology of Diabetes Interventions and Complications (EDIC) study." Jama **290**(16): 2159-67.

Eisenmann, D. M. (2005). "Wnt signaling." WormBook: 1-17.

Ellis, S. L., R. G. Naik, et al. (2008). "Use of continuous glucose monitoring in patients with type 1 diabetes." Curr Diabetes Rev **4**(3): 207-17.

Elshatory, Y. and L. Gan (2008). "The LIM-homeobox gene Islet-1 is required for the development of restricted forebrain cholinergic neurons." J Neurosci **28**(13): 3291-7.

Emamaullee, J. A., A. M. Shapiro, et al. (2006). "Neonatal porcine islets exhibit natural resistance to hypoxia-induced apoptosis." Transplantation **82**(7): 945-52.

Epple, A. and J. E. Brinn (1987). The comparative physiology of the pancreatic islets. Berlin ; New York, Springer-Verlag.

Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." Nature **292**: 154-6.

Fandrich, F., X. Lin, et al. (2002). "Preimplantation-stage stem cells induce long-term allogeneic graft acceptance without supplementary host conditioning." Nat Med **8**(2): 171-8.

Farkas, G., Z. Z. Fulop, et al. (1997). "Long-term function of fetal islet grafting prevents secondary diabetic complications." Transplant Proc **29**(1-2): 743-4.

Faustman, D. L., S. D. Tran, et al. (2006). "Comment on papers by Chong et al., Nishio et al., and Suri et al. on diabetes reversal in NOD mice." Science **314**(5803): 1243; author reply 1243.

Fioretto, P., M. W. Steffes, et al. (1998). "Reversal of lesions of diabetic nephropathy after pancreas transplantation." N Engl J Med **339**(2): 69-75.

Fiorina, P., F. Folli, et al. (2003). "Long-term beneficial effect of islet transplantation on diabetic macro-/microangiopathy in type 1 diabetic kidney-transplanted patients." Diabetes Care **26**(4): 1129-36.

Frank, A. M., C. F. Barker, et al. (2005). "Comparison of whole organ pancreas and isolated islet transplantation for type 1 diabetes." Adv Surg **39**: 137-63.

Freeman, H. and R. D. Cox (2006). "Type-2 diabetes: a cocktail of genetic discovery." Hum Mol Genet **15 Spec No 2**: R202-9.

Fujikawa, T., S. H. Oh, et al. (2005). "Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells." Am J Pathol **166**(6): 1781-91.

Fujikura, J., K. Hosoda, et al. (2006). "Notch/Rbp-j signaling prevents premature endocrine and ductal cell differentiation in the pancreas." Cell Metab **3**(1): 59-65.

Fujita, Y., A. T. Cheung, et al. (2004). "Harnessing the gut to treat diabetes." Pediatr Diabetes **5 Suppl 2**: 57-69.

Fukuda, K., Y. Tanigawa, et al. (1998). "cFKBP/SMAP; a novel molecule involved in the regulation of smooth muscle differentiation." Development **125**(18): 3535-42.

Gasa, R., C. Mrejen, et al. (2004). "Proendocrine genes coordinate the pancreatic islet differentiation program in vitro." Proc Natl Acad Sci U S A **101**(36): 13245-50.

Gavrieli, Y., Y. Sherman, et al. (1992). "Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation." J Cell Biol **119**(3): 493-501.

Gbenle, G. O. and E. O. Akinrimisi (1984). "A calcium dependent endoribonuclease from Trypanosoma congolense cytoplasm." Mol Biochem Parasitol **12**(1): 15-24.

Gerich, J. E. (1999). "Is insulin resistance the principal cause of type 2 diabetes?" Diabetes Obes Metab **1**(5): 257-63.

Gerrish, K., J. C. Van Velkinburgh, et al. (2004). "Conserved transcriptional regulatory domains of the pdx-1 gene." Mol Endocrinol **18**(3): 533-48.

Gershengorn, M. C., A. A. Hardikar, et al. (2004). "Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells." Science **306**(5705): 2261-4.

Gierl, M. S., N. Karoulias, et al. (2006). "The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells." Genes Dev **20**(17): 2465-78.

Gittes, G. K. and P. E. Galante (1993). "A culture system for the study of pancreatic organogenesis." J Tiss Cult Meth **15**: 23-28.

Gittes, G. K. and W. J. Rutter (1992). "Onset of cell-specific gene expression in the developing mouse pancreas." Proc Natl Acad Sci U S A **89**(3): 1128-32.

Godard, C. M. and K. W. Jones (1980). "Improved method for detection of cellular transcripts by in situ hybridization: detection of poly (A) sequences in individual cells." Histochemistry **65**(3): 291-300.

Gomez, E. J., M. E. Hernando Perez, et al. (2008). "The INCA system: a further step towards a telemedical artificial pancreas." IEEE Trans Inf Technol Biomed **12**(4): 470-9.

Govil, S. and C. W. Imrie (1999). "Value of splenic preservation during distal pancreatectomy for chronic pancreatitis." Br J Surg **86**(7): 895-8.

Gradwohl, G., A. Dierich, et al. (2000). "neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas." Proc Natl Acad Sci U S A **97**(4): 1607-11.

Gray, D. W. (2001). "An overview of the immune system with specific reference to membrane encapsulation and islet transplantation." Ann N Y Acad Sci **944**: 226-39.

Greschat, S., J. Schira, et al. (2008). "Unrestricted somatic stem cells from human umbilical cord blood can be differentiated into neurons with a dopaminergic phenotype." Stem Cells Dev **17**(2): 221-32.

Grille, S. J., A. Bellacosa, et al. (2003). "The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines." Cancer Res **63**(9): 2172-8.

Grompe, M. (2002). "Adult versus embryonic stem cells: it's still a tie." Mol Ther **6**(3): 303-5.

Gruessner, A. C. and D. E. Sutherland (2005). "Pancreas transplant outcomes for United States (US) and non-US cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) as of June 2004." Clin Transplant **19**(4): 433-55.

Gruss, P. (2003). "Human ES cells in Europe." Science **301**(5636): 1017.

Grzeschik, K. H. (1973). "Utilization of somatic cell hybrids for genetic studies in man." Humangenetik **19**(1): 1-40.

Gu, G., J. R. Brown, et al. (2003). "Direct lineage tracing reveals the ontogeny of pancreatic cell fates during mouse embryogenesis." Mechanisms of Development **120**(1): 35-43.

Gu, G., J. Dubauskaite, et al. (2002). "Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors." Development **129**(10): 2447-57.

Gupta, V., D. C. Wahoff, et al. (1997). "The defective glucagon response from transplanted intrahepatic pancreatic islets during hypoglycemia is transplantation site-determined." Diabetes **46**(1): 28-33.

Guz, Y., M. R. Montminy, et al. (1995). "Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny." Development **121**(1): 11-8.

Habener, J. F., D. M. Kemp, et al. (2005). "Minireview: transcriptional regulation in pancreatic development." Endocrinology **146**(3): 1025-34.

Hald, J., J. P. Hjorth, et al. (2003). "Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development." Dev Biol **260**(2): 426-37.

Hall, J. G., J. Kurtzberg, et al. (2005). "Partial splenectomy before a hematopoietic stem cell transplantation in children." J Pediatr Surg **40**(1): 221-7.

Halme, D. G. and D. A. Kessler (2006). "FDA regulation of stem-cell-based therapies." N Engl J Med **355**(16): 1730-5.

Hamburger, V. and H. L. Hamilton (1951). "A series of normal stages in the development of the chick embryo." J Morphology **88**: 49-92.

Hammerman, M. R. (2004). "Organogenesis of endocrine pancreas from transplanted embryonic anlagen." Transpl Immunol **12**(3-4): 249-58.

Hanafusa, T., A. Miyazaki, et al. (1990). "Examination of islets in the pancreas biopsy specimens from newly diagnosed type 1 (insulin-dependent) diabetic patients." Diabetologia **33**(2): 105-11.

Hardikar, A. A., B. Marcus-Samuels, et al. (2003). "Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates." Proc Natl Acad Sci U S A **100**(12): 7117-22.

Hargrave, M., J. Bowles, et al. (2006). "In situ hybridization of whole-mount embryos." Methods Mol Biol **326**: 103-13.

Harrison, K. A., J. Thaler, et al. (1999). "Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice." Nat Genet **23**(1): 71-5.

Hart, A., S. Papadopoulou, et al. (2003). "Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells." Dev Dyn **228**(2): 185-93.

Hatano, M., C. W. Roberts, et al. (1991). "Deregulation of a homeobox gene, HOX11, by the t(10;14) in T cell leukemia." Science **253**(5015): 79-82.

Hathout, E., J. Lakey, et al. (2003). "Islet transplant: an option for childhood diabetes?" Arch Dis Child **88**(7): 591-4.

Hauge-Evans, A. C., P. E. Squires, et al. (1999). "Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets." Diabetes **48**(7): 1402-8.

Haumaitre, C., E. Barbacci, et al. (2005). "Lack of TCF2/vHNF1 in mice leads to pancreas agenesis." Proc Natl Acad Sci U S A **102**(5): 1490-5.

Hebrok, M. (2003). "Hedgehog signaling in pancreas development." Mech Dev **120**(1): 45-57.

Hebrok, M., S. K. Kim, et al. (1998). "Notochord repression of endodermal Sonic hedgehog permits pancreas development." Genes Dev **12**(11): 1705-13.

Hebrok, M., S. K. Kim, et al. (2000). "Regulation of pancreas development by hedgehog signaling." Development **127**(22): 4905-13.

Hecksher-Sorensen, J., R. P. Watson, et al. (2004). "The splanchnic mesodermal plate directs spleen and pancreatic laterality, and is regulated by Bapx1/Nkx3.2." Development **131**(19): 4665-75.

Heinze, E. and A. Thon (1983). "Honeymoon period in insulin-dependent diabetes mellitus." Pediatrician **12**(4): 208-12.

Heiser, P. W., J. Lau, et al. (2006). "Stabilization of beta-catenin impacts pancreas growth." Development **133**(10): 2023-32.

Heit, J. J. and S. K. Kim (2004). "Embryonic stem cells and islet replacement in diabetes mellitus." Pediatr Diabetes **5 Suppl 2**: 5-15.

Heller, R. S., M. Jenny, et al. (2005). "Genetic determinants of pancreatic epsilon-cell development." Dev Biol **286**(1): 217-24.

Heller, R. S., T. Klein, et al. (2003). "Expression of Wnt, Frizzled, sFRP, and DKK genes in adult human pancreas." Gene Expr **11**(3-4): 141-7.

Hellerström, C. and K. Asplund (1966). " The two types of A-cells in the pancreatic islets of snakes." Cell and Tissue Research **70**(1): 68-80.

Hering, B. J., M. Wijkstrom, et al. (2006). "Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates." Nat Med **12**(3): 301-3.

Herrera, P. L., J. Huarte, et al. (1991). "Embryogenesis of the murine endocrine pancreas; early expression of pancreatic polypeptide gene." Development **113**(4): 1257-65.

Herzer, U., A. Crocoll, et al. (1999). "The Wilms tumor suppressor gene wt1 is required for development of the spleen." Curr Biol **9**(15): 837-40.

Hess, D., L. Li, et al. (2003). "Bone marrow-derived stem cells initiate pancreatic regeneration." Nat Biotechnol **21**(7): 763-70.

Hidai, H., R. Bardales, et al. (1998). "Cloning of capsulin, a basic helix-loop-helix factor expressed in progenitor cells of the pericardium and the coronary arteries." Mech Dev **73**(1): 33-43.

Hillier, L. W., W. Miller, et al. (2004). "Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution." Nature **432**(7018): 695-716.

Hirshberg, B., K. I. Rother, et al. (2003). "Islet transplantation: where do we stand now?" Diabetes Metab Res Rev **19**(3): 175-8; discussion 175.

Hisaoka, M., J. Haratake, et al. (1993). "Pancreatic morphogenesis and extracellular matrix organization during rat development." Differentiation **53**(3): 163-72.

Hoffmann-Fezer, G., H. Rodt, et al. (1977). "Anatomical distribution of T and B lymphocytes identified by immunohistochemistry in the chicken spleen." Int Arch Allergy Appl Immunol **55**(1-6): 86-95.

Holland, A. M., M. A. Hale, et al. (2002). "Experimental control of pancreatic development and maintenance." Proc Natl Acad Sci U S A **99**(19): 12236-41.

Hope, K. M., P. O. Tran, et al. (2004). "Regulation of alpha-cell function by the beta-cell in isolated human and rat islets deprived of glucose: the "switch-off" hypothesis." Diabetes **53**(6): 1488-95.

Hori, J., T. F. Ng, et al. (2003). "Neural progenitor cells lack immunogenicity and resist destruction as allografts." Stem Cells **21**(4): 405-16.

Hori, Y., X. Gu, et al. (2005). "Differentiation of insulin-producing cells from human neural progenitor cells." PLoS Med **2**(4): e103.

Houard, N., G. G. Rousseau, et al. (2003). "HNF-6-independent differentiation of mouse embryonic stem cells into insulin-producing cells." Diabetologia **46**(3): 378-85.

Huang, H. P., M. Liu, et al. (2000). "Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3." Mol Cell Biol **20**(9): 3292-307.

Huangfu, D., R. Maehr, et al. (2008). "Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds." Nat Biotechnol **26**(7): 795-7.

Hughes, S. J. (1994). "The role of reduced glucose transporter content and glucose metabolism in the immature secretory responses of fetal rat pancreatic islets." Diabetologia **37**(2): 134-40.

Hutchins, R. R., R. S. Hart, et al. (2002). "Long-term results of distal pancreatectomy for chronic pancreatitis in 90 patients." Ann Surg **236**(5): 612-8.

Ianus, A., G. G. Holz, et al. (2003). "In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion." J Clin Invest **111**(6): 843-50.

ICGSC (2004). "Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution (International Chicken Genome Sequencing Consortium)." Nature **432**(7018): 695-716.

Inzunza, J., S. Sahlen, et al. (2004). "Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation." Mol Hum Reprod **10**(6): 461-6.

Ishihara, H., P. Maechler, et al. (2003). "Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells." Nat Cell Biol **5**(4): 330-5.

Jacquemin, P., S. M. Durviaux, et al. (2000). "Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3." Mol Cell Biol **20**(12): 4445-54.

Jacquemin, P., C. E. Pierreux, et al. (2003). "Cloning and embryonic expression pattern of the mouse Onecut transcription factor OC-2." Gene Expr Patterns **3**(5): 639-44.

Jacquemin, P., H. Yoshitomi, et al. (2006). "An endothelial-mesenchymal relay pathway regulates early phases of pancreas development." Dev Biol **290**(1): 189-99.

Jaenisch, R. and A. Meissner (2006). "Politically correct human embryonic stem cells?" N Engl J Med **354**(11): 1208-9; author reply 1208-9.

Jayanthi, N. V., A. M. Rowan-Hull, et al. (2005). "The importance of pancreatic embryonic epithelium for mesenchyme-to-epithelial transition during islet development." Transplant Proc **37**(8): 3485-6.

Jensen, J. (2004). "Gene regulatory factors in pancreatic development." Dev Dyn **229**(1): 176-200.

Jetton, T. L., B. Everill, et al. (2008). "Enhanced beta-cell mass without increased proliferation following chronic mild glucose infusion." Am J Physiol Endocrinol Metab **294**(4): E679-87.

Jiang, J., M. Au, et al. (2007). "Generation of insulin-producing islet-like clusters from human embryonic stem cells." Stem Cells **25**(8): 1940-53.

Jirillo, E., M. L. Mastronardi, et al. (2003). "The immunocompromised host: immune alterations in splenectomized patients and clinical implications." Curr Pharm Des **9**(24): 1918-23.

Johnson, K. L. and D. W. Bianchi (2004). "Fetal cells in maternal tissue following pregnancy: what are the consequences?" Hum Reprod Update **10**(6): 497-502.

Johnson, K. L., J. L. Nelson, et al. (2001). "Fetal cell microchimerism in tissue from multiple sites in women with systemic sclerosis." Arthritis Rheum **44**(8): 1848-54.

Jonckheere, N., E. Mayes, et al. (2008). "Analysis of mPygo2 mutant mice suggests a requirement for mesenchymal Wnt signaling in pancreatic growth and differentiation." Dev Biol **318**(2): 224-35.

Jonsson, J., L. Carlsson, et al. (1994). "Insulin-promoter-factor 1 is required for pancreas development in mice." Nature **371**(6498): 606-9.

Kanai-Azuma, M., Y. Kanai, et al. (2002). "Depletion of definitive gut endoderm in Sox17-null mutant mice." Development **129**(10): 2367-79.

Kang, M., N. Kalra, et al. (2007). "Image guided percutaneous splenic interventions." Eur J Radiol **64**(1): 140-6.

Kanno, T., S. O. Gopel, et al. (2002). "Cellular function in multicellular system for hormone-secretion: electrophysiological aspect of studies on alpha-, beta- and delta-cells of the pancreatic islet." Neurosci Res **42**(2): 79-90.

Kanzler, B. and T. N. Dear (2001). "Hox11 acts cell autonomously in spleen development and its absence results in altered cell fate of mesenchymal spleen precursors." Dev Biol **234**(1): 231-43.

Karlsson, O., S. Thor, et al. (1990). "Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain." Nature **344**(6269): 879-82.

Kawaguchi, Y., B. Cooper, et al. (2002). "The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors." Nat Genet **32**(1): 128-34.

Keller, G., C. Wall, et al. (1998). "Overexpression of HOX11 leads to the immortalization of embryonic precursors with both primitive and definitive hematopoietic potential." Blood **92**(3): 877-87.

Kelly, W. D., R. C. Lillehei, et al. (1967). "Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy." Surgery **61**(6): 827-37.

Kendall, D. M., A. U. Teuscher, et al. (1997). "Defective glucagon secretion during sustained hypoglycemia following successful islet allo- and autotransplantation in humans." Diabetes **46**(1): 23-7.

Kennedy, M. A., R. Gonzalez-Sarmiento, et al. (1991). "HOX11, a homeobox-containing T-cell oncogene on human chromosome 10q24." Proc Natl Acad Sci U S A **88**(20): 8900-4.

Keymeulen, B., G. Korbutt, et al. (1996). "Long-term metabolic control by rat islet grafts depends on the composition of the implant." Diabetes **45**(12): 1814-21.

Kharroubi, I., L. Ladriere, et al. (2004). "Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress." Endocrinology **145**(11): 5087-96.

Khoursheed, M., F. Al-Sayegh, et al. (2004). "Laparoscopic splenectomy for hematological disorders." Med Princ Pract **13**(3): 122-5.

Kim, B. M., G. Buchner, et al. (2005). "The stomach mesenchymal transcription factor Barx1 specifies gastric epithelial identity through inhibition of transient Wnt signaling." Dev Cell **8**(4): 611-22.

Kim, B. M., I. Miletich, et al. (2007). "Independent functions and mechanisms for homeobox gene Barx1 in patterning mouse stomach and spleen." Development **134**(20): 3603-13.

Kim, J. Y., D. Kim, et al. (2005). "MHC expression in a human adult stem cell line and its down-regulation by hCMV US gene transfection." Int J Biochem Cell Biol **37**(1): 69-78.

Kim, S. K., M. Hebrok, et al. (2000). "Activin receptor patterning of foregut organogenesis." Genes Dev **14**(15): 1866-71.

Kim, S. K., M. Hebrok, et al. (1997). "Notochord to endoderm signaling is required for pancreas development." Development **124**(21): 4243-52.

Kim, S. K., M. Hebrok, et al. (1997). "Pancreas development in the chick embryo." Cold Spring Harb Symp Quant Biol **62**: 377-83.

Kim, S. K. and D. A. Melton (1998). "Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor." Proc Natl Acad Sci U S A **95**(22): 13036-41.

Kim, S. K., L. Selleri, et al. (2002). "Pbx1 inactivation disrupts pancreas development and in Ipf1-deficient mice promotes diabetes mellitus." Nat Genet **30**(4): 430-5.

Kimmelstiel, P. and C. Wilson (1936). "Benign and malignant hypertension and nephrosclerosis: clinical and pathological study." Am J Pathol **12**: 45-82.

King, H., R. E. Aubert, et al. (1998). "Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections." Diabetes Care **21**(9): 1414-31.

Kinkel, M. D., S. C. Eames, et al. (2008). "Cdx4 is required in the endoderm to localize the pancreas and limit beta-cell number." Development **135**(5): 919-29.

Kispert, A., S. Vainio, et al. (1998). "Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney." Development **125**(21): 4225-34.

Kleinman, R., G. Ohning, et al. (1994). "Regulatory role of intraislet somatostatin on insulin secretion in the isolated perfused human pancreas." Pancreas **9**(2): 172-8.

Klomp, G. F., S. H. Ronel, et al. (1979). "Hydrogels for encapsulation of pancreatic islet cells." Trans Am Soc Artif Intern Organs **25**: 74-6.

Kloppel, G., C. R. Drenck, et al. (1984). "Morphometric evidence for a striking B-cell reduction at the clinical onset of type 1 diabetes." Virchows Arch A Pathol Anat Histopathol **403**(4): 441-52.

Kobayashi, N. (2008). "Bioartificial pancreas for the treatment of diabetes." Cell Transplant **17**(1-2): 11-7.

Kobayashi, T., G. Harb, et al. (2006). "Immune mechanisms associated with the rejection of encapsulated neonatal porcine islet xenografts." Xenotransplantation **13**(6): 547-59.

Koblas, T., S. M. Harman, et al. (2005). "The application of umbilical cord blood cells in the treatment of diabetes mellitus." Rev Diabet Stud **2**(4): 228-34.

Kodama, S., M. Davis, et al. (2005). "Diabetes and stem cell researchers turn to the lowly spleen." Sci Aging Knowledge Environ **2005**(3): pe2.

Kodama, S., M. Davis, et al. (2005). "Regenerative medicine: a radical reappraisal of the spleen." Trends Mol Med **11**(6): 271-6.

Kodama, S., W. Kuhtreiber, et al. (2003). "Islet regeneration during the reversal of autoimmune diabetes in NOD mice." Science **302**(5648): 1223-7.

Koehler, K., T. Franz, et al. (2000). "Hox11 is required to maintain normal Wt1 mRNA levels in the developing spleen." Dev Dyn **218**(1): 201-6.

Korac, P., M. Jones, et al. (2005). "Application of the FICTION technique for the simultaneous detection of immunophenotype and chromosomal abnormalities in routinely fixed, paraffin wax embedded bone marrow trephines." J Clin Pathol **58**(12): 1336-8.

Koya, V., S. Lu, et al. (2008). "Reversal of streptozotocin-induced diabetes in mice by cellular transduction with recombinant pancreatic transcription factor pancreatic duodenal homeobox-1: a novel protein transduction domain-based therapy." Diabetes **57**(3): 757-69.

Kramer, B., A. Andrew, et al. (1987). "The effect of pancreatic mesenchyme on the differentiation of endocrine cells from gastric endoderm." Development **100**(4): 661-71.

Kramer, B., D. Manning, et al. (2002). "Absolute numbers versus proportions in the assessment of differentiation of beta-cells in the embryonic avian pancreas." Eur J Morphol **40**(3): 153-9.

Krapp, A., M. Knofler, et al. (1998). "The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas." Genes Dev **12**(23): 3752-63.

Kumar, M., N. Jordan, et al. (2003). "Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate." Dev Biol **259**(1): 109-22.

Kume, S. (2005). "The molecular basis and prospects in pancreatic development." Dev Growth Differ **47**(6): 367-74.

Kume, S. (2005). "Stem-cell-based approaches for regenerative medicine." Dev Growth Differ **47**(6): 393-402.

Larsen, J. L., C. W. Colling, et al. (2004). "Pancreas transplantation improves vascular disease in patients with type 1 diabetes." Diabetes Care **27**(7): 1706-11.

Lassman, M. N., R. T. O'Brien, et al. (1974). "Endocrine evaluation in thalassemia major." Ann N Y Acad Sci **232**(0): 226-37.

Le Douarin, N. (1969). "Particularites du noyau interphasique chez la caille Japonaise (Coturnix coturnix japonica)." Bulletin Biologique de la France et de la Belgique **103**(3): 435-52.

Leahy, J. L. (2005). "Pathogenesis of type 2 diabetes mellitus." Arch Med Res **36**(3): 197-209.

Lear, P. V., N. V. Jayanthi, et al. (2004). "Foregut Mesenchyme Contributes Cells to Islets during Pancreatic Development in a 3-Dimensional Avian Model." Organogenesis **1**(2): 45-51.

Lechner, A., A. L. Nolan, et al. (2005). "Redifferentiation of insulin-secreting cells after in vitro expansion of adult human pancreatic islet tissue." Biochem Biophys Res Commun **327**(2): 581-8.

Lee, B. W., S. H. Tan, et al. (1985). "Glucose tolerance test and insulin levels in children with transfusion-dependent thalassaemia." Ann Trop Paediatr **5**(4): 215-8.

Lee, C. S., N. J. Sund, et al. (2005). "Foxa2 is required for the differentiation of pancreatic alpha-cells." Dev Biol **278**(2): 484-95.

Lee, J., D. J. Han, et al. (2008). "In vitro differentiation of human adipose tissue-derived stem cells into cells with pancreatic phenotype by regenerating pancreas extract." Biochem Biophys Res Commun **375**(4): 547-51.

Lee, J. C., S. B. Smith, et al. (2001). "Regulation of the pancreatic pro-endocrine gene neurogenin3." Diabetes **50**(5): 928-36.

Lee, J. I., R. Nishimura, et al. (2008). "A newly developed immunoisolated bioartificial pancreas with cell sheet engineering." Cell Transplant **17**(1-2): 51-9.

Lee, T. C., N. R. Barshes, et al. (2006). "The effect of whole organ pancreas transplantation and PIT on diabetic complications." Curr Diab Rep **6**(4): 323-7.

Lee, T. C., N. R. Barshes, et al. (2005). "The effect of pancreatic islet transplantation on progression of diabetic retinopathy and neuropathy." Transplant Proc **37**(5): 2263-5.

Lettice, L. A., L. A. Purdie, et al. (1999). "The mouse bagpipe gene controls development of axial skeleton, skull, and spleen." Proc Natl Acad Sci U S A **96**(17): 9695-700.

Li, H., S. Arber, et al. (1999). "Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9." Nat Genet **23**(1): 67-70.

Li, L., M. L. Baroja, et al. (2004). "Human embryonic stem cells possess immune-privileged properties." Stem Cells **22**(4): 448-56.

Li, Y., R. Zhang, et al. (2007). "Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells." J Cell Physiol **211**(1): 36-44.

Li, Z., P. Manna, et al. (2004). "Multifaceted pancreatic mesenchymal control of epithelial lineage selection." Dev Biol **269**(1): 252-63.

Lieberman, S., E. Libson, et al. (2003). "Imaging-guided percutaneous splenic biopsy using a 20- or 22-gauge cutting-edge core biopsy needle for the diagnosis of malignant lymphoma." AJR Am J Roentgenol **181**(4): 1025-7.

Linning, K. D., M. H. Tai, et al. (2004). "Redox-mediated enrichment of self-renewing adult human pancreatic cells that possess endocrine differentiation potential." Pancreas **29**(3): e64-76.

Lints, T. J., L. M. Parsons, et al. (1993). "Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants." Development **119**(2): 419-31.

Liu, J., Z. Liu, et al. (2005). "Islet-1 expression and its colocalization with luteinising hormone, thyroid-stimulating hormone and oestrogen receptor alpha in the developing pituitary gland of the sheep foetus." J Neuroendocrinol **17**(12): 773-80.

Logan, C., R. J. Wingate, et al. (1998). "Tlx-1 and Tlx-3 homeobox gene expression in cranial sensory ganglia and hindbrain of the chick embryo: markers of patterned connectivity." J Neurosci **18**(14): 5389-402.

Lohr, J., B. Knoechel, et al. (2005). "T-cell tolerance and autoimmunity to systemic and tissue-restricted self-antigens." Immunol Rev **204**: 116-27.

Lohr, M. and G. Kloppel (1987). "Residual insulin positivity and pancreatic atrophy in relation to duration of chronic type 1 (insulin-dependent) diabetes mellitus and microangiopathy." Diabetologia **30**(10): 757-62.

Lonyai, A., S. Kodama, et al. (2008). "The promise of Hox11+ stem cells of the spleen for treating autoimmune diseases." Horm Metab Res **40**(2): 137-46.

Louz, D., H. E. Bergmans, et al. (2008). "Reappraisal of biosafety risks posed by PERVs in xenotransplantation." Rev Med Virol **18**(1): 53-65.

Lu, J., P. Chang, et al. (2000). "The basic helix-loop-helix transcription factor capsulin controls spleen organogenesis." Proc Natl Acad Sci U S A **97**(17): 9525-30.

Lu, J., J. A. Richardson, et al. (1998). "Capsulin: a novel bHLH transcription factor expressed in epicardial progenitors and mesenchyme of visceral organs." Mech Dev **73**(1): 23-32.

Lumelsky, N., O. Blondel, et al. (2001). "Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets." Science **292**(5520): 1389-94.

Ma, W., K. Rogers, et al. (2002). "Effects of different fixatives on beta-galactosidase activity." J Histochem Cytochem **50**(10): 1421-4.

MacDonald, P. E. and P. Rorsman (2006). "Oscillations, intercellular coupling, and insulin secretion in pancreatic beta cells." PLoS Biol **4**(2): e49.

Macfarlane, W. M., T. M. Frayling, et al. (2000). "Missense mutations in the insulin promoter factor-1 gene predispose to type 2 diabetes." J Clin Invest **106**(5): 717.

Maedler, K. (2008). "Beta cells in type 2 diabetes - a crucial contribution to pathogenesis." Diabetes Obes Metab **10**(5): 408-20.

Maffi, P., E. Angeli, et al. (2005). "Minimal focal steatosis of liver after islet transplantation in humans: a long-term study." Cell Transplant **14**(10): 727-33.

Makrin, V., S. Avital, et al. (2008). "Laparoscopic splenectomy for solitary splenic tumors." Surg Endosc **22**(9): 2009-12.

Mandel, T. E. (1999). "Fetal islet xenotransplantation in rodents and primates." J Mol Med **77**(1): 155-60.

Manuel, D. G. and S. E. Schultz (2004). "Health-related quality of life and health-adjusted life expectancy of people with diabetes in Ontario, Canada, 1996-1997." Diabetes Care **27**(2): 407-14.

Marchetti, P., S. Del Guerra, et al. (2004). "Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin." J Clin Endocrinol Metab **89**(11): 5535-41.

Martin, G. R. (1981). "Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma cell lines." *Natl Acad Sci USA* **78**: 7634-8.

Martinez-Ramirez, A., J. C. Cigudosa, et al. (2004). "Simultaneous detection of the immunophenotypic markers and genetic aberrations on routinely processed paraffin sections of lymphoma samples by means of the FICTION technique." Leukemia **18**(2): 348-53.

Mathews, V., P. T. Hanson, et al. (2004). "Recruitment of bone marrow-derived endothelial cells to sites of pancreatic beta-cell injury." Diabetes **53**(1): 91-8.

Matsumoto, S., K. Okumura, et al. (2007). "Isolation of tissue progenitor cells from duct-ligated salivary glands of swine." Cloning Stem Cells **9**(2): 176-90.

Matsushita, S. (1995). "Fate mapping study of the splanchnopleural mesoderm of the 1.5-day-old chick embryo." Roux's Arch Dev Biol **204**: 392-399.

McClish, R. D. and J. A. Eglitis (1969). "Distribution of the A and B cells of the islets (Langerhans) in the duck pancreas." Ohio J Sci **69**: 285-293.

McConnaha, S. (2005). "Blessed are the pluripotent: New Testament guidance for the embryonic stem cell debate." Natl Cathol Bioeth Q **5**(4): 707-15.

Mellitzer, G., S. Bonne, et al. (2006). "IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas." Embo J **25**(6): 1344-52.

Melton, D. A. (2006). "Reversal of type 1 diabetes in mice." N Engl J Med **355**(1): 89-90.

Mezey, E., A. Nagy, et al. (2003). "Comment on "Failure of bone marrow cells to transdifferentiate into neural cells in vivo"." Science **299**(5610): 1184; author reply 1184.

Miettinen, P. J., M. Huotari, et al. (2000). "Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors." Development **127**(12): 2617-27.

Miralles, F., T. Battelino, et al. (1998). "TGF-beta plays a key role in morphogenesis of the pancreatic islets of Langerhans by controlling the activity of the matrix metalloproteinase MMP-2." J Cell Biol **143**(3): 827-36.

Miralles, F., L. Lamotte, et al. (2006). "Interplay between FGF10 and Notch signalling is required for the self-renewal of pancreatic progenitors." Int J Dev Biol **50**(1): 17-26.

Miyazaki, S., E. Yamato, et al. (2004). "Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells from embryonic stem cells." Diabetes **53**(4): 1030-7.

Monti, P., M. Scirpoli, et al. (2008). "Islet transplantation in patients with autoimmune diabetes induces homeostatic cytokines that expand autoreactive memory T cells." J Clin Invest **118**(5): 1806-14.

Moscona, A. (1990). "Anatomy of the pancreas and langerhans islets in snakes and lizards." The Anatomical Record **227**: 232-244.

Muraca, S., P. G. Chait, et al. (2001). "US-guided core biopsy of the spleen in children." Radiology **218**(1): 200-6.

Naito, A. T., I. Shiojima, et al. (2006). "Developmental stage-specific biphasic roles of Wnt/beta-catenin signaling in cardiomyogenesis and hematopoiesis." Proc Natl Acad Sci U S A **103**(52): 19812-7.

Nakajima-Nagata, N., T. Sakurai, et al. (2004). "In vitro induction of adult hepatic progenitor cells into insulin-producing cells." Biochem Biophys Res Commun **318**(3): 625-30.

Nakaya, Y., S. Kuroda, et al. (2004). "Mesenchymal-epithelial transition during somitic segmentation is regulated by differential roles of Cdc42 and Rac1." Dev Cell **7**(3): 425-38.

Nanji, S. A. and A. M. Shapiro (2004). "Islet transplantation in patients with diabetes mellitus: choice of immunosuppression." BioDrugs **18**(5): 315-28.

Nanji, S. A. and A. M. Shapiro (2006). "Advances in pancreatic islet transplantation in humans." Diabetes Obes Metab **8**(1): 15-25.

Nathan, D. M., P. A. Cleary, et al. (2005). "Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes." N Engl J Med **353**(25): 2643-53.

Navarro, X., D. E. Sutherland, et al. (1997). "Long-term effects of pancreatic transplantation on diabetic neuropathy." Ann Neurol **42**(5): 727-36.

Nishio, J., J. L. Gaglia, et al. (2006). "Islet recovery and reversal of murine type 1 diabetes in the absence of any infused spleen cell contribution." Science **311**(5768): 1775-8.

Noguchi, H., S. Matsumoto, et al. (2006). "Immunosuppression for islet transplantation." Acta Medica Okayama **60**(2): 71-6.

Oakley, J. (2002). "Democracy, embryonic stem cell research, and the Roman Catholic church." J Med Ethics **28**(4): 228; discussion 229-31.

Oh, S. H., T. M. Muzzonigro, et al. (2004). "Adult bone marrow-derived cells trans-differentiating into insulin-producing cells for the treatment of type I diabetes." Lab Invest **84**(5): 607-17.

Oh, S. P. and E. Li (2002). "Gene-dosage-sensitive genetic interactions between inversus viscerum (iv), nodal, and activin type IIB receptor (ActRIIB) genes in asymmetrical patterning of the visceral organs along the left-right axis." Dev Dyn **224**(3): 279-90.

Ohlsson, H., K. Karlsson, et al. (1993). "IPF1, a homeodomain-containing transactivator of the insulin gene." Embo J **12**(11): 4251-9.

Okabayashi, T. and K. Hanazaki (2008). "Overwhelming postsplenectomy infection syndrome in adults - A clinically preventable disease." World J Gastroenterol **14**(2): 176-9.

Okuno, M., K. Minami, et al. (2007). "Generation of insulin-secreting cells from pancreatic acinar cells of animal models of type 1 diabetes." Am J Physiol Endocrinol Metab **292**(1): E158-65.

Omori, T., T. Nishida, et al. (2006). "A study of the xenoantigenicity of neonatal porcine islet-like cell clusters (NPCC) and the efficiency of adenovirus-mediated DAF (CD55) expression." Xenotransplantation **13**(5): 455-64.

Pabst, O., R. Zweigerdt, et al. (1999). "Targeted disruption of the homeobox transcription factor Nkx2-3 in mice results in postnatal lethality and abnormal development of small intestine and spleen." Development **126**(10): 2215-25.

Paeratakul, U., P. R. De Stasio, et al. (1988). "A fast and sensitive method for detecting specific viral RNA in mammalian cells." J Virol **62**(4): 1132-5.

Pang, K., C. Mukonoweshuro, et al. (1994). "Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas." Proc Natl Acad Sci U S A **91**(20): 9559-63.

Park, H. W., Y. M. Chae, et al. (1992). "Morphogenic development of the pancreas in the staged human embryo." Yonsei Med J **33**(2): 104-8.

Passa, P. (2002). "Diabetes trends in Europe." Diabetes Metab Res Rev **18 Suppl 3**: S3-8.

Patterson, K. D., T. A. Drysdale, et al. (2000). "Embryonic origins of spleen asymmetry." Development **127**(1): 167-75.

Paty, B. W., E. A. Ryan, et al. (2002). "Intrahepatic islet transplantation in type 1 diabetic patients does not restore hypoglycemic hormonal counterregulation or symptom recognition after insulin independence." Diabetes **51**(12): 3428-34.

Paueksakon, P., M. P. Revelo, et al. (2002). "Microangiopathic injury and augmented PAI-1 in human diabetic nephropathy." Kidney Int **61**(6): 2142-8.

Percival, A. C. and J. M. Slack (1999). "Analysis of pancreatic development using a cell lineage label." Experimental Cell Research **247**(1): 123-32.

Perry, J. M., O. F. Harandi, et al. (2008). "Maintenance of the BMP4 dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling." Blood.

Pictet, R. L., W. R. Clark, et al. (1972). "An ultrastructural analysis of the developing embryonic pancreas." Dev Biol **29**(4): 436-67.

Pipeleers, D. and Z. Ling (1992). "Pancreatic beta cells in insulin-dependent diabetes." Diabetes Metab Rev **8**(3): 209-27.

Piters, E., E. Boudin, et al. (2008). "Wnt signaling: a win for bone." Arch Biochem Biophys **473**(2): 112-6.

Pomp, A., M. Gagner, et al. (2005). "Laparoscopic splenectomy: a selected retrospective review." Surg Laparosc Endosc Percutan Tech **15**(3): 139-43.

Pontoglio, M., S. Sreenan, et al. (1998). "Defective insulin secretion in hepatocyte nuclear factor 1alpha-deficient mice." J Clin Invest **101**(10): 2215-22.

Porksen, N., S. Munn, et al. (1994). "Coordinate pulsatile insulin secretion by chronic intraportally transplanted islets in the isolated perfused rat liver." J Clin Invest **94**(1): 219-27.

Purrello, F. and D. Pipeleers (1995). "Transplantation in diabetes: a cell biological problem." J Endocrinol Invest **18**(4): 311-9.

Pyzdrowski, K. L., D. M. Kendall, et al. (1992). "Preserved insulin secretion and insulin independence in recipients of islet autografts." N Engl J Med **327**(4): 220-6.

Quaggin, S. E., L. Schwartz, et al. (1999). "The basic-helix-loop-helix protein pod1 is critically important for kidney and lung organogenesis." Development **126**(24): 5771-83.

Rackley, R. R., A. M. Flenniken, et al. (1993). "Expression of the Wilms' tumor suppressor gene WT1 during mouse embryogenesis." Cell Growth Differ **4**(12): 1023-31.

Raju, K., S. Tang, et al. (1993). "Characterization and developmental expression of Tlx-1, the murine homolog of HOX11." Mech Dev **44**(1): 51-64.

Ramalho-Santos, M., D. A. Melton, et al. (2000). "Hedgehog signals regulate multiple aspects of gastrointestinal development." Development **127**(12): 2763-72.

Rawdon, B. B. (1998). "Morphogenesis and differentiation of the avian endocrine pancreas, with particular reference to experimental studies on the chick embryo." Microsc Res Tech **43**(4): 292-305.

Reed, G. H. and C. T. Wittwer (2004). "Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis." Clin Chem **50**(10): 1748-54.

Renard, E. and P. Schaepelynck-Belicar (2007). "Implantable insulin pumps. A position statement about their clinical use." Diabetes Metab **33**(2): 158-66.

Rickels, M. R., M. H. Schutta, et al. (2007). "Glycemic thresholds for activation of counterregulatory hormone and symptom responses in islet transplant recipients." J Clin Endocrinol Metab **92**(3): 873-9.

Rickels, M. R., M. H. Schutta, et al. (2005). "Islet cell hormonal responses to hypoglycemia after human islet transplantation for type 1 diabetes." Diabetes **54**(11): 3205-11.

Robb, L., L. Mifsud, et al. (1998). "epicardin: A novel basic helix-loop-helix transcription factor gene expressed in epicardium, branchial arch myoblasts, and mesenchyme of developing lung, gut, kidney, and gonads." Dev Dyn **213**(1): 105-13.

Roberts, C. W., J. R. Shutter, et al. (1994). "Hox11 controls the genesis of the spleen." Nature **368**(6473): 747-9.

Roberts, C. W., A. M. Sonder, et al. (1995). "Development expression of Hox11 and specification of splenic cell fate." Am J Pathol **146**(5): 1089-101.

Roberts, D. J., D. M. Smith, et al. (1998). "Epithelial-mesenchymal signaling during the regionalization of the chick gut." Development **125**(15): 2791-801.

Robertson, S. A., A. M. Rowan-Hull, et al. (2008). "The spleen--a potential source of new islets for transplantation?" J Pediatr Surg **43**(2): 274-8.

Roche, E., R. Ensenat-Waser, et al. (2007). "Insulin-producing cells from embryonic stem cells experimental considerations." Methods Mol Biol **407**: 295-309.

Rood, P. P., R. Bottino, et al. (2006). "Facilitating physiologic self-regeneration: a step beyond islet cell replacement." Pharm Res **23**(2): 227-42.

Rorsman, P. and E. Renstrom (2003). "Insulin granule dynamics in pancreatic beta cells." Diabetologia **46**(8): 1029-45.

Rose, M. I., D. C. Brown, et al. (1999). "Effects of microgravity on the embryonic pancreas." In Vitro Cell Dev Biol Anim **35**(10): 560-3.

Rose, M. I., C. A. Crisera, et al. (1999). "Epithelio-mesenchymal interactions in the developing mouse pancreas: morphogenesis of the adult architecture." J Pediatr Surg **34**(5): 774-9; discussion 780.

Rose, V., T. Izukawa, et al. (1975). "Syndromes of asplenia and polysplenia. A review of cardiac and non-cardiac malformations in 60 cases withspecial reference to diagnosis and prognosis." Br Heart J **37**(8): 840-52.

Rosen, M., F. Brody, et al. (2002). "Outcome of laparoscopic splenectomy based on hematologic indication." Surg Endosc **16**(2): 272-9.

Rother, K. I. and D. M. Harlan (2004). "Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus." J Clin Invest **114**(7): 877-83.

Rowan-Hull, A. M., R. Rao, et al. (In Press). "ISL-1 is induced in stomach mesenchyme in the presence of pancreatic epithelia." Journal Pediatric Surgery.

Ruhnke, M., H. Ungefroren, et al. (2005). "Differentiation of in vitro-modified human peripheral blood monocytes into hepatocyte-like and pancreatic islet-like cells." Gastroenterology **128**(7): 1774-86.

Ruiz-Tovar, J., J. Perez-de Oteyza, et al. (2007). "[Laparoscopic splenectomy: 7-year experience]." Cir Cir **75**(4): 287-91.

Rulifson, I. C., S. K. Karnik, et al. (2007). "Wnt signaling regulates pancreatic beta cell proliferation." Proc Natl Acad Sci U S A **104**(15): 6247-52.

Ryan, E. A., J. R. Lakey, et al. (2002). "Successful islet transplantation: continued insulin reserve provides long-term glycemic control." Diabetes **51**(7): 2148-57.

Ryan, E. A., B. W. Paty, et al. (2005). "Five-year follow-up after clinical islet transplantation." Diabetes **54**(7): 2060-9.

Ryu, S., S. Kodama, et al. (2001). "Reversal of established autoimmune diabetes by restoration of endogenous beta cell function." J Clin Invest **108**(1): 63-72.

Sadler, T. W. and J. Langman (1995). Langman's medical embryology. Baltimore, Williams & Wilkins.

Saitoh, K., E. Yamato, et al. (2007). "Both Pdx-1 and NeuroD1 genes are requisite for the maintenance of insulin gene expression in ES-derived differentiated cells." Diabetes Res Clin Pract **77 Suppl 1**: S138-42.

Santana, A., R. Ensenat-Waser, et al. (2006). "Insulin-producing cells derived from stem cells: recent progress and future directions." J Cell Mol Med **10**(4): 866-83.

Sasaki, K. and G. Matsumura (1988). "Spleen lymphocytes and haemopoiesis in the mouse embryo." J Anat **160**: 27-37.

Saudek, C. D., R. M. Hemm, et al. (1977). "Abnormal glucose tolerance in beta-thalassemia major." Metabolism **26**(1): 43-52.

Schwitzgebel, V. M., D. W. Scheel, et al. (2000). "Expression of neurogenin3 reveals an islet cell precursor population in the pancreas." Development **127**(16): 3533-42.

Seaberg, R. M., S. R. Smukler, et al. (2004). "Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages." Nat Biotechnol **22**(9): 1115-24.

Selleri, L., M. J. Depew, et al. (2001). "Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation." Development **128**(18): 3543-57.

Servitja, J. M. and J. Ferrer (2004). "Transcriptional networks controlling pancreatic development and beta cell function." Diabetologia **47**(4): 597-613.

Shamblott, M. J. and G. O. Clark (2004). "Cell therapies for type 1 diabetes mellitus." Expert Opin Biol Ther **4**(3): 269-77.

Shapiro, A. M., J. R. Lakey, et al. (2000). "Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen." N Engl J Med **343**(4): 230-8.

Shen, W. F., C. P. Chang, et al. (1996). "Hox homeodomain proteins exhibit selective complex stabilities with Pbx and DNA." Nucleic Acids Res **24**(5): 898-906.

Shostak, S. (2006). "(Re)defining stem cells." Bioessays **28**(3): 301-8.

Silecchia, G., L. Raparelli, et al. (2005). "Laparoscopic splenectomy for non-traumatic diseases." Minerva Chir **60**(5): 363-74.

Simon, H. G. and C. J. Tabin (1993). "Analysis of Hox-4.5 and Hox-3.6 expression during newt limb regeneration: differential regulation of paralogous Hox genes suggest different roles for members of different Hox clusters." Development **117**(4): 1397-407.

Slack, J. M. (1995). "Developmental biology of the pancreas." Development **121**(6): 1569-80.

Slack, J. M., G. Lin, et al. (2008). "The Xenopus tadpole: a new model for regeneration research." Cell Mol Life Sci **65**(1): 54-63.

Slack, J. M. and D. Tosh (2001). "Transdifferentiation and metaplasia--switching cell types." Curr Opin Genet Dev **11**(5): 581-6.

Smith, P. H. (1974). "Pancreatic islets of the coturnix quail. A light and electron microscopic study with special reference to the islet organ of the splenic lobe." Anat Rec **178**: 567-85.

Soeldner, J. S., M. Tuttleman, et al. (1985). "Insulin-dependent diabetes mellitus and autoimmunity: islet-cell autoantibodies, insulin autoantibodies, and beta-cell failure." N Engl J Med **313**(14): 893-4.

Solomon, M. F., W. A. Kuziel, et al. (2004). "The contribution of chemokines and chemokine receptors to the rejection of fetal proislet allografts." Cell Transplant **13**(5): 503-14.

Soltesz, G., C. C. Patterson, et al. (2007). "Worldwide childhood type 1 diabetes incidence--what can we learn from epidemiology?" Pediatr Diabetes **8 Suppl 6**: 6-14.

Soon-Shiong, P., R. E. Heintz, et al. (1994). "Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation." Lancet **343**(8903): 950-1.

Soria, B., E. Roche, et al. (2000). "Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice." Diabetes **49**(2): 157-62.

Spooner, B. S., P. Hardman, et al. (1994). "Gravity in mammalian organ development: differentiation of cultured lung and pancreas rudiments during spaceflight." J Exp Zool **269**(3): 212-22.

SRTR (2007). "Scientific Registry of Transplant Recipients annual report ".

Stadtfeld, M., K. Brennand, et al. (2008). "Reprogramming of pancreatic beta cells into induced pluripotent stem cells." Curr Biol **18**(12): 890-4.

Stafford, D., A. Hornbruch, et al. (2004). "A conserved role for retinoid signaling in vertebrate pancreas development." Dev Genes Evol **214**(9): 432-41.

Stainier, D. (2006). "No stem cell is an islet (yet)." N Engl J Med **354**(5): 521-3.

Stern, C. D. (2004). Gastrulation : from cells to embryo. Cold Spring Harbor, N.Y. ; [Great Britain], Cold Spring Harbor Laboratory Press.

Stoffers, D. A., J. Ferrer, et al. (1997). "Early-onset type-II diabetes mellitus (MODY4) linked to IPF1." Nat Genet **17**(2): 138-9.

Streeter, G. L. (1942). "Developmental Horizons in Human Embryos. Description of age group XI, 13 to 20 nomites, and age group XII, 21 to 29 somites." Contr Embryol Carneg Instn **30**: 211-245.

Streeter, G. L. (1948). "Developmental Horizons in Human Embryos. Description of age groups XV, XVI, XVII, and XVIII, being the third issue of a survey of the Carnegie Collection. ." Contr Embryol Carneg Instn **32**: 133-203.

Streit, A. and C. D. Stern (2001). "Combined whole-mount in situ hybridization and immunohistochemistry in avian embryos." Methods **23**(4): 339-44.

Sugaya, K. (2003). "Potential use of stem cells in neuroreplacement therapies for neurodegenerative diseases." Int Rev Cytol **228**: 1-30.

Sukegawa, A., T. Narita, et al. (2000). "The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium." Development **127**(9): 1971-80.

Sun, B., K. H. Roh, et al. (2007). "Induction of human umbilical cord blood-derived stem cells with embryonic stem cell phenotypes into insulin producing islet-like structure." Biochem Biophys Res Commun **354**(4): 919-23.

Suri, A., B. Calderon, et al. (2006). "Immunological reversal of autoimmune diabetes without hematopoietic replacement of beta cells." Science **311**(5768): 1778-80.

Sussel, L., J. Kalamaras, et al. (1998). "Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells." Development **125**(12): 2213-21.

Suzuki, A., H. Nakauchi, et al. (2003). "Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells." Proc Natl Acad Sci U S A **100**(9): 5034-9.

Takahashi, K., K. Tanabe, et al. (2007). "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." Cell **131**(5): 861-72.

Tam, A., S. Krishnamurthy, et al. (2008). "Percutaneous image-guided splenic biopsy in the oncology patient: an audit of 156 consecutive cases." J Vasc Interv Radiol **19**(1): 80-7.

Tamborlane, W. V. (2006). "Fulfilling the promise of insulin pump therapy in childhood diabetes." Pediatr Diabetes **7 Suppl 4**: 4-10.

Tang, D. Q., L. Z. Cao, et al. (2006). "Role of Pax4 in Pdx1-VP16-mediated liver-to-endocrine pancreas transdifferentiation." Lab Invest **86**(8): 829-41.

Tateishi, K., J. He, et al. (2008). "Generation of insulin-secreting islet-like clusters from human skin fibroblasts." J Biol Chem(Sep 9 [Epub ahead of print]).

Taupin, P. (2006). "Derivation of embryonic stem cells for cellular therapy: challenges and new strategies." Med Sci Monit **12**(4): RA75-8.

Teague, W. J., N. V. Jayanthi, et al. (2005). "Foregut mesenchyme contributes cells to pancreatic acini during embryonic development in a chick-quail chimera model." Pediatr Surg Int **21**(3): 138-42.

Teague, W. J., A. M. Rowan-Hull, et al. (2006). "The competency of foregut mesenchyme in islet mesenchyme-to-epithelial transition during embryonic development." J Pediatr Surg **41**(2): 347-51.

Teague, W. J., A. M. Rowan-Hull, et al. (2007). "Pancreatic alpha-cell differentiation by mesenchyme-to-epithelial transition: implications for cell-based therapies in children." J Pediatr Surg **42**(1): 153-9.

Thomson, J. A., J. Itskovitz-Eldor, et al. (1998). "Embryonic stem cell lines derived from human blastocysts." Science **282**(5391): 1145-7.

Thor, S., J. Ericson, et al. (1991). "The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat." Neuron **7**(6): 881-9.

Timper, K., D. Seboek, et al. (2006). "Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells." Biochem Biophys Res Commun **341**(4): 1135-40.

Tissier-Seta, J. P., M. L. Mucchielli, et al. (1995). "Barx1, a new mouse homeodomain transcription factor expressed in cranio-facial ectomesenchyme and the stomach." Mech Dev **51**(1): 3-15.

Todorov, I., K. Omori, et al. (2006). "Generation of human islets through expansion and differentiation of non-islet pancreatic cells discarded (pancreatic discard) after islet isolation." Pancreas **32**(2): 130-8.

Tosh, D. and J. M. Slack (2002). "How cells change their phenotype." Nat Rev Mol Cell Biol **3**(3): 187-94.

Treff, N. R., R. K. Vincent, et al. (2006). "Differentiation of embryonic stem cells conditionally expressing neurogenin 3." Stem Cells **24**(11): 2529-37.

Tribioli, C. and T. Lufkin (1999). "The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen." Development **126**(24): 5699-711.

Trucco, M., A. Casu, et al. (2007). "The pig as the donor of pancreatic islets for men." Vet Res Commun **31 Suppl 1**: 27-33.

Ullmann, U., C. Gilles, et al. (2008). "GSK-3-specific inhibitor-supplemented hESC medium prevents the epithelial-mesenchymal transition process and the up-regulation of matrix metalloproteinases in hESCs cultured in feeder-free conditions." Mol Hum Reprod **14**(3): 169-79.

Vaca, P., G. Berna, et al. (2008). "Nicotinamide induces differentiation of embryonic stem cells into insulin-secreting cells." Exp Cell Res **314**(5): 969-74.

Valdes-Gonzalez, R. A., L. M. Dorantes, et al. (2005). "Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study." Eur J Endocrinol **153**(3): 419-27.

Van Belle, T. and M. von Herrath (2008). "Immunosuppression in islet transplantation." J Clin Invest **118**(5): 1625-8.

van Rooijen, N., E. Claassen, et al. (1989). "Cytological basis of immune functions of the spleen. Immunocytochemical characterization of lymphoid and non-lymphoid cells involved in the 'in situ' immune response." Prog Histochem Cytochem **19**(3): 1-71.

Volk, B. W. and E. R. Arquilla (1985). The Diabetic pancreas. New York, Plenum Medical Book Co.

Waldman, J. D., A. Rosenthal, et al. (1977). "Sepsis and congenital asplenia." J Pediatr **90**(4): 555-9.

Wang, G. S., L. Rosenberg, et al. (2005). "Tubular complexes as a source for islet neogenesis in the pancreas of diabetes-prone BB rats." Lab Invest **85**(5): 675-88.

Wang, J., G. Cortina, et al. (2006). "Mutant neurogenin-3 in congenital malabsorptive diarrhea." N Engl J Med **355**(3): 270-80.

Wang, L., Y. M. Liu, et al. (2005). "Comparison of F-actin fluorescent labeling methods in pollen tubes of Lilium davidii." Plant Cell Rep **24**(5): 266-70.

Wang, M. and D. J. Drucker (1994). "The LIM domain homeobox gene isl-1: conservation of human, hamster, and rat complementary deoxyribonucleic acid sequences and expression in cell types of nonneuroendocrine lineage." Endocrinology **134**(3): 1416-22.

Wang, M. J. and P. Gegenheimer (1990). "Substrate masking: binding of RNA by EGTA-inactivated micrococcal nuclease results in artifactual inhibition of RNA processing reactions." Nucleic Acids Res **18**(22): 6625-31.

Wang, Q., L. Elghazi, et al. (2008). "Ghrelin is a novel target of Pax4 in endocrine progenitors of the pancreas and duodenum." Dev Dyn **237**(1): 51-61.

Way, J. C. and M. Chalfie (1988). "mec-3, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in C. elegans." Cell **54**(1): 5-16.

Wei, J. P., T. S. Zhang, et al. (2003). "Human amnion-isolated cells normalize blood glucose in streptozotocin-induced diabetic mice." Cell Transplant **12**(5): 545-52.

Weinstein, D. C., A. Ruiz i Altaba, et al. (1994). "The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo." Cell **78**(4): 575-88.

Weir, G. C. (2004). "Can we make surrogate beta-cells better than the original?" *Semin Cell Dev Biol* **15**: 347-57.

Weissman, I. L. (2000). "Translating stem and progenitor cell biology to the clinic: barriers and opportunities." Science **287**(5457): 1442-6.

Wells, J. M. and D. A. Melton (2000). "Early mouse endoderm is patterned by soluble factors from adjacent germ layers." Development **127**(8): 1563-72.

White, E. M. and M. A. Gorovsky (1988). "Localization and expression of mRNA for a macronuclear-specific histone H2A variant (hv1) during the cell cycle and conjugation of Tetrahymena thermophila." Mol Cell Biol **8**(11): 4780-6.

White, P. and E. Waskow (1948). "Arteriosclerosis in childhood diabetes." Proc Am Diab assn **8**: 141-149.

Wild, S., G. Roglic, et al. (2004). "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030." Diabetes Care **27**(5): 1047-53.

Wilkinson, D. G. (1992). In situ hybridization : a practical approach. Oxford ; New York, IRL Press at Oxford University Press.

Williams, P. W. (1894). "Notes on diabetes treated with extract and by grafts of sheep's pancreas." *Br Med J* **2**: 1303-1304.

Wilting, J., M. Papoutsi, et al. (2001). "Development of the avian lymphatic system." Microsc Res Tech **55**(2): 81-91.

Wu, F., M. Jagir, et al. (2004). "Long-term correction of hyperglycemia in diabetic mice after implantation of cultured human cells derived from fetal pancreas." Pancreas **29**(1): e23-9.

Xu, X., J. D'Hoker, et al. (2008). "Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas." Cell **132**(2): 197-207.

Yamada, S., K. Terada, et al. (2005). "Differentiation of adult hepatic stem-like cells into pancreatic endocrine cells." Cell Transplant **14**(9): 647-53.

Yang, L., S. Li, et al. (2002). "In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells." Proc Natl Acad Sci U S A **99**(12): 8078-83.

Yassine, F., B. Fedecka-Bruner, et al. (1989). "Ontogeny of the chick embryo spleen--a cytological study." Cell Differ Dev **27**(1): 29-45.

Yasugi, S. and T. Mizuno (2008). "Molecular analysis of endoderm regionalization." Dev Growth Differ **50 Suppl 1**: S79-96.

Yin, D., J. Tao, et al. (2006). "Recovery of islet beta-cell function in streptozotocin-induced diabetic mice: an indirect role for the spleen." Diabetes **55**(12): 3256-63.

Yokoo, T., T. Ohashi, et al. (2005). "Human mesenchymal stem cells in rodent whole-embryo culture are reprogrammed to contribute to kidney tissues." Proc Natl Acad Sci U S A **102**(9): 3296-300.

Yokoyama, T., N. G. Copeland, et al. (1993). "Reversal of left-right asymmetry: a situs inversus mutation." Science **260**(5108): 679-82.

Yorston, D. (2003). "Retina picture quiz: questions (figs. 1 to 6)." Community Eye Health **16**(46): 27-8.

Zalzman, M., S. Gupta, et al. (2003). "Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells." Proc Natl Acad Sci U S A **100**(12): 7253-8.

Zapata, A. G. and E. L. Cooper (1990). The immune system : comparative histophysiology. Chichester ; New York, Wiley.

Zhang, J. H., J. L. Liu, et al. (2006). "LIM homeodomain proteins Islet-1 and Lim-3 expressions in the developing pineal gland of chick embryo by immunohistochemistry." J Pineal Res **41**(3): 247-54.

Zhao, Y., H. Wang, et al. (2006). "Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics." Exp Cell Res **312**(13): 2454-64.

Zhou, Q., J. Brown, et al. (2008). "In vivo reprogramming of adult pancreatic exocrine cells to beta-cells." Nature **455**(7213): 627-32.

Zulewski, H., E. J. Abraham, et al. (2001). "Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes." Diabetes **50**(3): 521-33.

Zuppinger, K., A. Teuscher, et al. (1975). "[Frequency of juvenile diabetes mellitus in the Canton Bern (Switzerland)]." Schweiz Med Wochenschr **105**(38): 1218-23.